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**ATHEROGENESIS AND ATHEROSCLEROSIS IN
PRIMARY ANTIPHOSPHOLIPID SYNDROME**

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DEDICATION

This thesis is dedicated to my dear friend Giulio with whom I shared the happiness of youth, the goliardy of our school days, the enthusiasm for our life projects and the emotions of his first rugby matches. It is during one of these that tragedy befell and he passed away without ever reaching the try-line. His friendship lived on within me and I would like to consider this late accomplishment as the try that he never scored.

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ABSTRACT

Background

In the late seventies the term “Haematological Stress Syndrome” defined some haematological abnormalities appearing in the course of acute and chronic disorders, such as raised plasma levels of fibrinogen (FNG) and factor VIII, reduced fibrinolytic activity and hyperviscosity. In the early nineties the “Membrane stress syndrome hypothesis” proposed the unification of the concepts of haematological stress syndrome with those of oxidation, inflammation and immune activation to explain the pathogenesis of the antiphospholipid syndrome (APS)

Antiphospholipid antibodies, coagulation, fibrinolysis and thrombosis

This chapter investigated the occurrence of the “Haematological Stress Syndrome” and thrombosis in 144 participants positive for aPL detected by clotting and immune tests. Among the clotting assays for the detection of lupus anticoagulant, dilute Russell's viper venom time better correlated with a history of venous thrombosis than activated partial thromboplastin time ($p<0.0002$ vs $p<0.009$) and was the only test correlated with a history of arterial thrombosis ($p<0.01$). By regression analysis, serum levels of IgG anticardiolipin antibodies (aCL) associated with the number of venous occlusions ($p<0.001$). With regards to FNG and von Willebrand factor (vWF), the former rose by 36% (95% CI; 21%, 53%) and the latter by 50% (95% CI; 29%, 75%) at the first venous occlusion and remained unchanged after subsequent occlusions. At variance FNG rose by 45% (95% CI; 31%, 60%) per arterial occlusion and vWF by 27% (95% CI; 10%, 47%) per arterial occlusion throughout.

The coagulation/fibrinolytic balance was cross-sectionally evaluated on 18 thrombotic PAPS patients, 18 subjects with persistence of idiopathic aPL and in healthy controls. Markers of thrombin generation prothrombin fragment 1+2 (F1+2), thrombin-antithrombin complex (TAT) and of fibrin turnover D-Dimer (D-D) were higher in thrombotic ($p=0.006$)

and non-thrombotic subjects ($p=0.0001$) than in controls as were those of D-D ($p<0.0001$ and $p=0.003$ respectively). TAT levels did not differ. Gender analysed data revealed blunted tPA release (hence a negative venous occlusion test) in thrombotic females but neither in thrombotic males ($p=0.01$) nor in asymptomatic subjects of either sex. Also, in both patient groups females had higher mean PAI than males ($p<0.0002$) and control females ($p<0.02$).

The activity of factor XIII (FXIIIa) was evaluated was evaluated in 29 patients with PAPS, 14 persistent carriers of aPL without thrombosis, 24 thrombotic patients with inherited thrombophilia, 28 healthy controls and 32 patients with mitral and aortic valve prosthesis as controls for FXIII only. FXIIIa was highest in PAPS ($p=0.001$), particularly in patients with multiple ($n=12$) than single occlusion ($p=0.02$) and in correlation with PAI ($p=0.003$) and FNG ($p=0.005$). Moreover FXIIIa was strongly associated with IgG aCL and IgG anti- β_2 GPI ($p=0.005$ for both) in the PAPS group and to a lesser degree in the aPL group (FXIIIa with IgG aCL, $p=0.02$, with IgG anti- β_2 GPI, $p=0.04$). Altogether these results indicate: 1) a differential relationship of aPL, vWF and FNG with venous and arterial thrombosis; 2) heightened thrombin generation, accelerated fibrin turnover and fibrinolysis abnormalities also in asymptomatic carriers of aPLs; 3) enhanced FXIIIa that may contribute to atherothrombosis via increased fibrin/fibrinogen cross-linking.

Lipid profile, lipid peroxidation and anti-lipoprotein antibodies in thrombotic primary antiphospholipid syndrome.

Given the atherogenic lipid profile of SLE, the same possibility was explored in PAPS by comparing high-density lipoprotein (HDL), low-density lipoprotein (LDL), total cholesterol (CHO), apolipoprotein AI (ApoAI), apolipoprotein B (ApoB), triglycerides (TG), anti-lipoprotein antibodies, beta-2-glycoprotein I complexed to oxidized low-density lipoprotein (oxLDL- β_2 GPI) and C-reactive protein (CRP) in 34 thrombotic PAPS patients compared to 36 thrombotic patients with inherited thrombophilia (IT), to 18 subjects persistently positive

for antiphospholipid antibodies (aPL) with no underlying autoimmune or non-autoimmune disorders and to 28 healthy controls. Average concentrations of HDL ($p<0.0001$), LDL ($p<0.0001$), CHO ($p=0.0002$), ApoAI ($p=0.002$) were lower in PAPS whereas average TRY was higher ($p=0.01$) than other groups. Moreover PAPS showed higher IgG anti-HDL ($p=0.01$) and IgG anti-ApoAI ($p<0.0001$) as well as greater average oxLDL- β_2 GPI ($p=0.001$) and CRP ($p=0.003$). Within PAPS, IgG anti-HDL correlated negatively to HDL ($p=0.001$) and CRP ($p=0.003$). Within PAPS, IgG anti-HDL correlated negatively to HDL ($p=0.004$) and was an independent predictor of oxLDL- β_2 GPI ($p=0.009$). HDL and ApoAI correlated negatively with CRP ($p=0.001$ and $p=0.007$, respectively). IgG anti-HDL may hamper the antioxidant and anti-inflammatory effect of HDL favouring low-grade inflammation and enhanced oxidation in thrombotic PAPS. Indeed plasma 8-epi-prostaglandin F2 α (a very specific marker of lipid peroxidation) was significantly higher in 10 patients with PAPS than 10 age and sex matched healthy subjects ($p=0.0002$) and strongly related to the titre of plasma IgG aCL ($r=0.89$, $p=0.0004$). Hence oxidative stress, a major player in atherogenesis, also characterises PAPS.

Nitric oxide and nitrative stress in thrombotic primary antiphospholipid syndrome.

Oxidative stress goes hand in hand with nitrative stress and to address the latter plasma nitrotyrosine (NT, marker of nitrative stress), nitrite (NO_2^-) and nitrate (NO_3^-) were measured in 46 thrombotic PAPS patients, 21 asymptomatic but persistent carriers of antiphospholipid antibodies (PCaPL), 38 patients with inherited thrombophilia (IT), 33 patients with systemic lupus erythematosus (SLE) and 29 healthy controls (CTR). Average crude NT was higher in PAPS and SLE ($p=0.01$) whereas average plasma NO_2^- was lower in PAPS and average NO_3^- highest in SLE ($p<0.0001$). In PAPS, IgG aCL titer and number of vascular occlusions negatively predicted NO_2^- , ($p=0.03$ and $p=0.001$, respectively) whereas arterial occlusions and smoking positively predicted NO_3^- ($p=0.05$ and $p=0.005$). Moreover CRP (an inflammatory marker) positively predicted NT ($p=0.004$). Nitric oxide metabolites relates to type and

number of vascular occlusions and to aPL titers, whereas oxidative stress relates to low grade inflammation and both phenomena may have implications for thrombosis and atherosclerosis in PAPS

Inflammation and immune activation in thrombotic primary antiphospholipid syndrome.

To investigate inflammation and immune activation in thrombotic PAPS high-sensitivity CRP (hs-CRP), serum amyloid A (SAA), oxLDL- β_2 GPI, CRP bound to oxLDL- β_2 GPI (CRP-oxLDL- β_2 GPI) (as inflammatory markers) neopterin (NPT) and soluble CD14 (sCD14) (as immune activation markers) were measured by ELISA in 41 PAPS patients, in 44 patients with inherited thrombophilia (IT) and 39 controls (CTR). Compared to other groups, PAPS presented with higher plasma concentrations of inflammatory, hs-CRP ($p=0.0004$), SAA ($p<0.01$), CRP-oxLDL- β_2 GPI ($p=0.0004$) and immune activation markers, NPT ($p<0.0001$) and sCD14 ($p=0.007$). By regression analysis SAA independently predicted thrombosis number ($p=0.003$) and NPT independently predicted thrombosis type (arterial, $p=0.03$) and number ($p=0.04$). These data confirm that low-grade inflammation and immune activation occur and relate to vascular features of PAPS.

Antiphospholipid antibodies, haemostatic variables and atherosclerosis in thrombotic primary antiphospholipid syndrome

To evaluate whether IgG aCL titre, haemostatic variables and the lipid profile bore any relationship to the intima media thickness (IMT) of carotid arteries high-resolution sonography was applied to the common carotid (CC), carotid bifurcation (CB) and internal carotid (IC) of 42 aPL subjects, 29 with primary thrombotic antiphospholipid syndrome and 13 with persistence of aPL in the absence of any underlying disorder. The following were measured: plasma FNG, vWF, PAI, homocysteine (HC), CHO, TG, HDL, LDL, platelet numbers and aCL of IgG and IgM isotype. By multiple regression analysis, IgG aCL titre independently predicted IMT at all carotid segments examined (p always <0.005). Plasma

FNG and HC independently predicted IMT at the CB ($p=0.001$ and $p<0.0001$, respectively) and IC ($p=0.03$ and $p<0.0001$, respectively). These data strongly support an atherogenic role for IgG aCL in patients with aPL in addition to traditional risk factors. The atherosclerosis hypothesis was investigated in an age and sex-matched case-double-control study including 49 thrombotic PAPS patients (18 M, 31 F, mean age 37 ± 11), 49 thrombotic patients for IT and 49 healthy subjects. Average IMT was always greater in PAPS than control patients (CC: $p=0.004$, CB: $p=0.013$, IC: $p=0.001$). By dividing participants into age tertiles the IMT was greater in the second (CC: $p=0.003$, CB: $p=0.023$, IC: $p=0.003$) and third tertiles (CC: $p=0.03$, CB: $p=0.004$, IC: $p=0.007$).

Conclusion

Coagulation activation, fibrinolysis depression, heightened fibrin turnover, oxidative and nitrate stress in parallel with low grade inflammation and immune activation characterise thrombotic PAPS: all these are early atherogenic processes and contribute to the demonstrated premature atherosclerosis that should be considered a clinical feature of PAPS.

RESUMO

Introdução

No final dos anos setenta algumas alterações hematológicas como níveis elevados de fibrinogénio (FNG) e factor VIII, diminuição da actividade fibrinolítica e hiperviscosidade foram agrupadas na definição “Síndrome de Stress Hematológico”.

Posteriormente, a hipótese da “Síndrome de Stress Membranar” veio propor a unificação dos conceitos de síndrome de stress hematológico, oxidação, inflamação e activação imunológica, numa tentativa de explicação da patogénese da síndrome de anticorpos antifosfolípidos (APS).

Anticorpos antifosfolípidos, coagulação, fibrinólise e trombose

Este capítulo aborda os conceitos da “Síndrome de stress hematológico” e trombose em 144 doentes com anticorpos antifosfolípidos (aPL), detectados por testes imunológicos e de coagulação. Entre os testes para detecção do anticoagulante lúpico (LA), o “tempo de veneno de cobra” (DRVVT) apresentou uma melhor correlação com a ocorrência de trombozes venosas que o tempo de tromboplastina parcial activado ($p < 0.0002$ vs $p < 0.009$) e foi o único teste em que se verificou uma correlação com a presença de trombozes arteriais ($p < 0.01$). A análise de regressão, após correcção para potenciais factores de confundimento, verificou uma associação entre os níveis séricos de anticorpos anti-cardiolipina (aCL) IgG e o número de trombozes venosas ($p < 0.001$). No que se refere ao FNG e factor de Von Willebrand (vWF), verificou-se uma elevação de 36% do primeiro (95% CI; 21%, 53%) e de 50% do segundo (95% CI; 29%, 75%) na primeira trombose venosa. Estes valores mantiveram-se inalterados nos eventos subsequentes. Em contraste, verificou-se uma subida de 45% do FNG (95% CI, 31%-60%) e de 27% do vWF (95% CI; 10%-47%) por cada evento trombótico arterial. O equilíbrio entre coagulação e fibrinólise foi avaliado de forma transversal em 18 doentes com PAPS, 18 doentes com aPL persistentemente elevados e em

controles saudáveis. Marcadores de geração de trombina como o fragmento 1+2 da protrombina (F1+2) e complexos trombina-anti-trombina (TAT) e da renovação da fibrina como os dímeros D (DD) estavam mais elevados em doentes com trombozes ($p=0.006$) e mesmo em doentes sem trombozes ($p=0.0001$) do que no grupo controlo, tal como os DD ($p<0.0001$ e $p=0.003$ respectivamente). Os valores de TAT não foram significativamente diferentes.

Níveis médios de proteína S livre mais baixos foram encontrados no grupo com PAPS ($p=0.0006$) e nos indivíduos sem trombozes ($p=0.002$) em comparação com os controlos saudáveis. Em ambos os grupos de estudo, os níveis de F1+2 eram mais elevados em doentes com níveis baixos de proteína S quando comparados com os indivíduos que apresentavam valores de proteína S normais ($p=0.01$). No que diz respeito ao género, observou-se uma redução do activador tissular do plasminogénio (rPA) (avaliado através do teste de oclusão venosa), em mulheres com PAPS (de 16.80 ± 0.79 para 21.3 ± 3.9 ng/ml, NS), mas não em homens com PAPS (de 18.2 ± 2.0 para 33.7 ± 4.9 ng/ml, $p=0.01$), nem nos doentes com anticorpos mas sem eventos trombóticos, independentemente do género.

Da mesma forma, em ambos os grupos de doentes, as mulheres apresentaram níveis de inibidor do activador de plasminogénio (PAI) mais elevados que os homens ($p<0.0002$) e que os controlos femininos ($p<0.02$).

A actividade do factor XIII (FXIIIa) foi avaliada em 29 doentes com PAPS, 14 portadores de anticorpos antifosfolípidos mas sem eventos trombóticos, 24 doentes com trombofilias hereditárias e trombozes, 28 controlos saudáveis e 32 doentes com próteses mitrales ou aórticas (utilizados apenas para controlo dos níveis de FXIII).

A FXIIIa estava mais elevada em doentes com PAPS ($p=0.001$), particularmente em doentes com mais de um evento trombótico ($158 \pm 45\%$ vs $118 \pm 38\%$; $p=0.02$, $n=12$) e correlacionou-se com os níveis de PAI ($p=0.003$) e FNG ($p=0.005$).

A FXIIIa estava ainda fortemente associada aos títulos de aCL IgG e anti- β_2 GPI IgG ($p=0.005$ para ambos) no grupo com PAPS e, embora de forma menos significativa, no grupo portador de anticorpos (aCL IgG, $p=0.02$; anti- β_2 GPI IgG, $p=0.04$).

Em conjunto estes resultados mostram: 1) uma relação diferencial de aPL, vWF e FNG com as trombozes arteriais e venosas; 2) o aumento da formação de trombina, o aumento da renovação da fibrina e a diminuição da actividade fibrinolítica ocorrem também nos portadores assintomáticos de aPL; 3) um papel central da deficiência de proteína S livre “adquirida” no risco trombótico dos doentes com APS; 4) um aumento da actividade do FXIII que poderá contribuir para aterotrombose através de um aumento das ligações cruzadas entre fibrina e fibrinogénio.

Perfil lipidico, peroxidação lipidica e anticorpos anti-lipoproteínas na síndrome de anticorpos antifosfolípidos

Estes aspectos foram estudados comparando os níveis de HDL, LDL, colesterol total (CHO), apolipoproteína AI (ApoAI), apolipoproteína B (ApoB), triglicéridos (TG), anticorpos anti-lipoproteínas, complexos beta-2-glicoproteína-I e LDL oxidada (oxLDL- β_2 GPI) e proteína C reactiva (CRP) em 34 doentes com PAPS, 36 doentes com trombofilias hereditárias e trombozes, 18 portadores persistentes de anticorpos antifosfolípidos e 28 controlos saudáveis.

Os níveis de HDL ($p<0.0001$), LDL ($p<0.0001$), CHO ($p=0.0002$) e ApoAI ($p=0.002$) foram mais baixos no grupo com PAPS, enquanto os TG foram mais elevados ($p=0.01$) que nos outros grupos. Doentes com PAPS mostraram ainda ter títulos mais elevados de anti-HDL IgG ($p=0.01$) e anti-ApoAI IgG ($p<0.0001$), e níveis superiores de oxLDL- β_2 GPI ($p=0.001$) e CRP ($p=0.003$).

Dentro do grupo com PAPS, os níveis de anti-HDL IgG correlacionaram-se negativamente com os valores de HDL ($p=0.004$) e foram predictores independentes dos

valores de oxLDL- β_2 GPI ($p=0.009$). HDL e ApoAI também se correlacionaram negativamente com a CRP ($p=0.001$ e $p=0.007$, respectivamente).

Anticorpos anti-HDL IgG podem bloquear os efeitos anti-oxidante e anti-inflamatório das HDL, favorecendo um estado de inflamação persistente e um aumento da oxidação no PAPS. De facto, a 8-epi-prostaglandina F2 α (um marcador específico de peroxidação lipídica) estava significativamente mais elevado em 10 doentes com PAPS quando comparados com um grupo igual controlado para sexo e idade (234 ± 56 pg/ml vs 72 ± 14 pg/ml, $p=0.0002$) e correlacionou-se directamente com os títulos de aCL IgG ($r=0.89$, $p=0.0004$). Estes dados confirmam o facto de o stress oxidativo ser um factor importante no PAPS.

Óxido nítrico (NO), stress nitrativo e PAPS

O stress oxidativo associa-se ao stress nitrativo e para estudar o segundo, avaliaram-se os níveis plasmáticos de nitrotirosina (NT), nitrito (NO_2^-) e nitrato (NO_3^-) em 46 doentes com PAPS, 21 portadores assintomáticos de anticorpos aPL, 38 doentes com trombofilias hereditárias (IT), 33 doentes com lupus sistémico (SLE) e 29 controlos saudáveis. Os níveis de NT foram mais elevados nos grupos com PAPS e SLE ($p=0.01$), enquanto que os níveis de NO_2^- foram mais baixos no PAPS e os de NO_3^- mais elevados no SLE ($p<0.0001$).

No grupo com PAPS, os títulos de aCL IgG e o número de oclusões vasculares foram predictores negativos dos níveis de NO_2^- , ($p=0.03$ e $p=0.001$, respectivamente), enquanto as oclusões arteriais e consumo de tabaco foram predictores positivos de NO_3^- ($p=0.05$ e $p=0.005$). Para além destes dados, os valores de CRP foram predictores positivos dos níveis de NT ($p=0.004$). Os metabolitos do NO estão associados ao tipo e número de trombooses vasculares e aos títulos de aPL, enquanto que o stress nitrativo se associa à inflamação de baixa intensidade persistente.

Inflamação e activação imunológica no PAPS

Para avaliar estes mecanismos em doentes com PAPS, foram medidos os níveis de CRP de alta sensibilidade (hs-CRP), amiloide sérico A (SAA), oxLDL- β_2 GPI e CRP ligada a oxLDL- β_2 GPI (CRP-oxLDL- β_2 GPI) (como marcadores inflamatórios), e neopterin (NPT) e CD14 solúvel (sCD14) (como marcadores de activação imunológica) em 41 doentes com PAPS, 44 doentes com IT e 39 controlos saudáveis. Comparado com os outros grupos, os doentes com PAPS apresentaram valores mais elevados de hs-CRP ($p=0.0004$), SAA ($p<0.01$), CRP-oxLDL- β_2 GPI ($p=0.0004$), NPT ($p<0.0001$) e sCD14 ($p=0.007$). A análise de regressão mostrou que o SAA é predictor independente do número de trombozes ($p=0.003$) e a NPT do tipo de trombose (arterial, $p=0.03$) e do número de eventos ($p=0.04$).

Anticorpos antifosfolípidos, variáveis hemostáticas e aterosclerose no PAPS

Para avaliar uma possível relação entre os títulos de aCL IgG, variáveis hemostáticas e perfil lipídico, e a espessura da íntima e da média da parede arterial (IMT), realizaram-se eco-dopplers da carótida comum (CC), bifurcação (B) e carótida interna (IC) em 42 doentes com aPL (29 com PAPS e 13 portadores de aPL sem trombozes). Foram avaliados: FNG, vWF, PAI, homocisteína (HC), CHO, TG, HDL, LDL, número de plaquetas e aCL (IgG e IgM). A análise de regressão mostrou que os níveis de aCL IgG são predictores independentes da IMT em todos os segmentos ($p<0.005$ para todos). FNG e HC foram predictores independentes da IMT na bifurcação ($p=0.001$ e $p<0.0001$, respectivamente) e na IC ($p=0.03$ e $p<0.0001$, respectivamente).

Estes dados suportam o papel pró-aterogénico dos aCL IgG em associação aos factores de risco tradicionais. Estes dados foram complementados com o estudo de 49 doentes com PAPS, 49 doentes com IT e 49 controlos saudáveis. A IMT média foi sempre maior no grupo do PAPS (CC: $p=0.004$; B: $p=0.013$; IC: $p=0.001$). Ao dividir os indivíduos estudados em tercios de acordo com a idade, a IMT era maior no segundo e terceiro tercios (CC: $p=0.003$; B:

p=0.023; IC: p=0.003) e (CC: p=0.03; B: p=0.004; IC: p=0.007), respectivamente. Desta forma confirma-se a existência de aterosclerose como manifestação integrante do síndrome de anticorpos antifosfolípidos primário.

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LIST OF ABBREVIATIONS

β_2 GPI: beta-2-glycoprotein-I

aCL: anticardiolipin

aPL: antiphospholipid

ApoA-I: apolipoprotein A-I

APS: antiphospholipid syndrome

aPTT: activated partial thromboplastin time

CHD: coronary artery disease

CL: cardiolipin

CRP: C-reactive protein

CVD: cardiovascular disease

D-D: dimer D

DRVVT: dilute Russel's viper venom time

DVT: deep vein thrombosis

eNOS: endothelial nitric oxide synthase

ET: endothelin

F1+2: prothrombin fragment 1+2

FNG: fibrinogen

FNT: fibronectin

f-PS: free protein S

FXIIIa: factor XIII activity

HC: homocysteine

HDL: high density lipoprotein

IMT: intima media thickness

iNOS: inducible nitric oxide synthase

IS: ischaemic stroke

IT: inherited thrombophilia

KCT: kaolin clotting time

LA: lupus anticoagulant

LDL: low density lipoprotein

MI: myocardial infarction

NO•: nitric oxide

NO₂⁻: nitrite

NO₃⁻: nitrate

NPT: neopterin

NT: nitrotyrosine

OxLDL: oxidated LDL

PAI: plasminogen activator inhibitor

PAPS: primary antiphospholipid syndrome

PON: paraoxonase

ROS: reactive oxygen species

SAA: serum amyloid A

SLE: systemic lupus erythematosus

TAC: total antioxidant capacity of plasma

TAT: thrombin-antithrombin complex

TG: tryglicerides

TNF- α :Tumor necrosis factor-alpha

tPA: tissue plasminogen activator

TxB₂: thromboxane B₂

vWF: von Willebrand factor

CHAPTER I

THE ANTIPHOSPHOLIPID SYNDROME: DIAGNOSIS, CLASSIFICATION AND CLINICAL MANIFESTATIONS

HISTORICAL MILESTONES

In the early '80s a syndrome characterized by thrombosis, thrombocytopenia and recurrent miscarriages was described in patients with systemic lupus erythematosus (SLE) who were found positive for antibodies to cardiolipin (CL). Anti CL antibodies (aCL) belong to the wider family of “antiphospholipid” antibodies (aPL) the specificity of which was initially attributed to negatively charged phospholipids. It became evident this was not the case and that the major aPL specificity was towards proteins bound to negatively charged phospholipids. The major antigenic target has been identified in beta-2-glycoprotein-I (β_2 GPI), a plasma protein of liver origin whose functions are well beyond those of coagulation regulation. The immunisation of different strains of mice with β_2 GPI induce the appearance of specific antibodies that have thrombogenic properties *in vitro* and *in vivo*, and more interestingly β_2 GPI immunisation of low density lipoprotein knockout mice induce the appearance of premature atherosclerosis.

Although the antiphospholipid syndrome (APS) was described in 1983, the existence of aCL and lupus anticoagulant (LA) was already known. In 1906 Wasserman (1) described a complement fixation test to detect “reagin” in the sera of syphilitic patients and in 1941 Pangborn (2) demonstrated that reagin bound to an antigen extracted from ox heart muscle. This antigenic extract was later named cardiolipin. In the following years Moore and Mohr (3) recognized that the “reagin test” was also positive in some patients who had never contracted a treponemal infection. By analysing these patients they identified a first group in whom the false positive “reagin” was a transient phenomenon, generally the result of an

infection, and a second group in which the false positive reaction was a persistent phenomenon. In this latter group there was a high prevalence of patients with SLE.

The first description of a circulating anticoagulant in patients with SLE was given by Conley and Hartmann (4) in 1952. Their patients showed a bleeding tendency, contrary to the increased thrombotic risk recognized by Bowie in 1963 (5). A decade later Feinstein and Rappaport (6) named the inhibitor “lupus anticoagulant”. This was also found in association with placental infarctions and recurrent miscarriages. In 1983 Harris and colleagues (7) first detected aPLs as aCL by a radioimmunoassay and then by an enzyme linked immunoassay (Elisa) (8). In 1990, three independent groups of scientists (9-11) discovered that the aCL detected by Elisa was not directed towards CL alone. Purified IgG from aCL positive patients failed to bind CL, unless a “cofactor” was present. The “cofactor” was identified as β_2 GPI, a plasma apolipoprotein with phospholipid binding properties involved in several steps of the coagulation pathway.

From the clinical perspective the independent existence of APS outside SLE had been suspected in the mid '80 (12, 13) and in the late '80s a multicenter study documented the major clinical and serologic characteristics of a primary APS. Essentially these patients suffered deep vein thromboses, often accompanied by pulmonary embolism, eventually complicated by thromboembolic pulmonary hypertension, arterial occlusions (ischaemic strokes and myocardial infarctions) or fetal loss; thrombocytopaenia, haemolytic anaemias and isolated Coombs positivity were also described. The negativity of antinuclear antibodies, antibodies against dsDNA and extractable nuclear antigens ENA was the main serologic features that discriminated them from SLE (14). These findings were confirmed in a later European survey (15). A survey performed ten years later revealed that patients with primary APS have little propensity to develop SLE (16).

BETA2-GLYCOPROTEIN I

β_2 GPI is a highly glycosylated protein synthesized in hepatocytes and circulates in the plasma at a concentration range between 50–500 mg/mL though approximately 40% associates with lipoproteins; this single chain protein consists of 326 amino acids and is arranged in five mutually homologous domains consisting of approximately 60 amino acids bridged by disulphide bonds to form five short consensus repeats called Sushi domains (17). β_2 GPI circulates in blood in a circular conformation that shields the epitope for aPL binding within domain I, but after interaction of domain V with anionic surfaces in vivo or with anionic phospholipids in vitro, the circular structure opens up, the cryptic epitope is exposed and aPL can bind to it (18).

Beta-2-glycoprotein I, antiphospholipid antibodies and thrombogenesis

β_2 GPI regulates several steps of the coagulation pathway, exerting both procoagulant and anticoagulant activities. β_2 GPI binds factor XI in vitro preventing activation of FXI to FXIa by thrombin and FXIIa. Proteolytic cleavage of the phospholipid binding domain of β_2 GPI abolishes its inhibition of FXI activation. β_2 GPI protects thrombin from heparin inactivation (19), modulates protein C activation, inhibits von Willebrand factor dependent platelet adhesion and aggregation (20) and stimulates fibrinolysis (21). Whenever aPL binds β_2 GPI the activity of the natural anticoagulant functions is lost and this effect, combined with the aPL induced over-expression of tissue factor on monocytes and endothelial cells lead to excessive thrombin generation. Thrombin promotes fibrinogen polymerisation, platelet activation and further endothelial cell activation with a shift from an anti-adhesive and anti-thrombotic phenotype to a pro-adhesive and pro-thrombotic phenotype. Finally enhanced thrombin generation coupled with aPL impairment of fibrinolysis causes increased fibrin turnover.

Beta-2-glycoprotein I, antiphospholipid antibodies and atherogenesis

Free radicals generated by endothelial cells and circulating neutrophils, monocytes and platelets may induce oxidative modifications within LDL (oxLDL) the uptake of which by mononuclear cells is mediated by their scavenger receptors and is followed by monocyte migration under the endothelial layer and then by their transformation in foam cells or lipid laden macrophages. During this process monocytes/macrophages release a number of inflammatory and chemotactic cytokines that contribute locally to the development of atherosclerosis. Immune-staining of human atherosclerotic lesions co-localized oxLDL with β_2 GPI. Indeed β_2 GPI may interact with oxLDL in an antioxidant fashion initially via electrostatic interactions that over time become covalent: this process takes place in the arterial intima of atherosclerotic lesions and produces stable and non-dissociable oxLDL β_2 GPI complexes (reviewed in 22). From the cellular point of view, after co-incubation of IgG anti- β_2 GPI antibodies with oxLDL and β_2 GPI, the monocyte/macrophage uptake and intracellular accumulation of oxLDL accelerates and leads to up-regulation and enhanced surface expression of scavenger (CD36) and Fc γ RI receptors that perpetuates this cycle. Therefore, while antibodies against β_2 GPI favor the development of thrombosis, antibodies against oxLDL β_2 GPI may favor the development of atherosclerosis (22).

DIAGNOSIS AND CLASSIFICATION OF THE ANTIPHOSPHOLIPID SYNDROME

Clinical diagnosis of antiphospholipid syndrome

The diagnosis of the APS relies on clinical and laboratory criteria. The clinical criteria include one or more clinical episodes of arterial, venous, or small-vessel thrombosis in any organ or tissue confirmed by adequate imaging or histopathology findings. Occlusions may involve either venous or arterial vessels in the brain, heart, lungs, abdomen or limbs.

Investigations should be instigated if any of these occur in younger individuals (males < 55 y; females < 65 y) or in the absence of other risk factors. With regards to pregnancy morbidity, the criteria include one or more late-term (>10 weeks' gestation) spontaneous abortions, one or more premature births of a morphologically healthy neonate at or before 34 weeks of gestation for severe preeclampsia or eclampsia or severe placental insufficiency; three or more unexplained, consecutive, spontaneous abortions before 10 weeks of gestation (23).

Clinical classification of antiphospholipid syndrome

The APS may appear in different settings: 1) as primary APS (PAPS) in patients with no underlying disorder but for the presence and persistence of aPLs and clinical manifestations, 2) in patients with definite SLE, 3) as secondary APS in a variety of other autoimmune and non-autoimmune conditions and as a “catastrophic” syndrome in patients with rapidly progressive multi-organ failure (23).

Laboratory diagnosis of antiphospholipid syndrome

The laboratory criteria include (1) medium to high levels of immunoglobulin (Ig) G or IgM aCL, (2) anti- β_2 GPI, or (3) lupus anticoagulant on at least 2 occasions at least 12 weeks apart. Although the detection principles of these assays are different, the common denominator of these three assays is that a positive result depends on the presence of β_2 GPI (24).

Immunological detection of antiphospholipid antibodies

Two different but established enzyme linked immune assays are available for the detection of aPL: in the first, the microplate is coated with CL as the target antigen (though there is a minute amount of β_2 GPI) in the second the microplate is coated with β_2 GPI as the target antigen. The former assay is more sensitive but less specific, whereas the latter is highly specific (24).

Lupus anticoagulant testing

Clotting assays for the identification of a LA rely on the ability of the immunoglobulin to interfere with phospholipid dependent stages of coagulation. Minimal criteria for the diagnosis of a LA are 1) prolongation of PL dependent coagulation test 2) discrimination between a clotting factor deficiency and circulating anticoagulant 3) confirmation of the lupus inhibitor activity of the anticoagulant. Common screening tests include kaolin clotting time (KCT), activated partial thromboplastin time (aPTT) and dilute Russell viper venom time (dRVVT). The KCT is highly sensitive, but lacks specificity and may be affected by residual platelets in plasma samples after centrifugation. The aPTT is widely used as a screening procedure, although its ability to detect LA varies according to the sensitivity of the reagent employed for the assay. The dRVVT is highly specific being associated with arterial thrombosis. Once a screening test shows prolongation of the clotting time, mixing studies using patient and normal plasma become mandatory. Correction of the prolonged clotting time suggests a clotting factor deficiency whereas failure of correction is diagnostic of an inhibitor. In this case a thrombin and a reptilase time must exclude the presence of heparin or an acquired dysfibrinogenaemia respectively. The antiphospholipid nature of the anticoagulant can be evaluated by several approaches. The inhibitor effect may be potentiated by decreasing the phospholipid concentration in the test systems, as in the dRVVT and the dilute aPTT. The inhibitor effect may be overcome by increasing the phospholipid concentration. This applies to the platelet or phospholipids neutralisation procedures in aPTT or dRVVT systems and to the KCT performed with low and high rabbit brain cephalin concentrations (25). Phospholipids with an altered configuration have been used to bypass the inhibitor effect of LA and a comparison between sensitive and insensitive reagents to LA has proven utility (26).

Laboratory classification of antiphospholipid syndrome

The previous immunological and coagulation assays allow a classification of the laboratory criteria into 4 categories: category IIa as lupus anticoagulant alone, IIb as aCL alone, IIc as anti- β_2 GPI alone and category I as any combination of the previous (23).

CLINICAL MANIFESTATIONS

aPL and venous thrombosis

In the setting of SLE, an early study from the '90s revealed LA as the strongest risk factor for venous thrombosis with an odds ratio of 6.6 (27) whereas a later meta-analysis from the same decade found an odds ratio of 5.6 for LA and 2.2 for aCL (28). The more recent LUMINA Study showed that 9% of SLE patients had at least one episode of venous thrombosis independently predicted by LA (29). Outside the autoimmune setting, the association of venous thrombosis with aPLs has been assessed in patients with venous thrombosis as well as in population-based prospective studies. A meta-analysis from the mid '90s found an overall odds ratio of 11.1 for LA whereas the odds ratio for aCL of any titer was approximately 1.6, and for high titer aCL was 3.2 (30). A later systematic review data from 25 primary studies including more than 7000 patients and controls, revealed LA as a strong risk factor for venous thrombosis with an odds ratios ranging from 5 to 16; a weaker association was found for aCL that did not reach statistical significance in about half of the studies reviewed (31).

aPL and ischemic stroke

Amongst arterial vessels the cerebral circulation is most often occluded in APS patients leading to ischemic stroke or transient ischemic attacks; the middle cerebral artery is prevalently affected (32). The Eurolupus study showed that ischaemic stroke was the initial

presentation in 29.9% of adults with APS (33) and a lower 18% presentation prevalence was derived from APS patients of Latin American origin (34). Another study detected a very high odds ratio of 43.1 for LA tests with regards to ischemic strokes in a population with a low positivity rate for LA (35). Ischaemic stroke is also the most frequent recurrent occlusive event and responsible for elevated mortality. aPL-associated ischaemic strokes accounted for 11.8% of these events in a predominantly young female cohort (36). Ischaemic stroke occurred also in 62% of the 250 patients in the European Catastrophic Antiphospholipid Antibody Syndrome (CAPS) registry and was the leading cause of death in 13% of the 114 deaths in this registry (37).

aPL and myocardial infarction

Myocardial infarction is a consistent feature of APS being the presenting manifestation in 2.8% (38) of patients and occurring in up to 5.5% of patients with APS (39) and vascular myocardial involvement is often asymptomatic (40).

Catastrophic antiphospholipid syndrome

A small number of patients with elevated titres of aPLs and/or LA may develop a syndrome characterized by the sudden onset of widespread vascular occlusions and rapidly progressive multiorgan failure. Fatality rate is high, and death may ensue as a result of irreversible renal, cardiac, pulmonary or cerebral damage as mentioned earlier (37). Acute respiratory distress syndrome appeared in some patients who presented with pulmonary microvascular thrombosis, alveolar hemorrhage and capillaritis. A disorder resembling thrombotic thrombocytopenic purpura developed in a small number of patients with SLE and aPLs. Major characteristics were microangiopathic hemolytic anemia with the presence of schistocytes, thrombocytopenia, renal dysfunction and central nervous system disease.

Pregnancy loss

Recurrent spontaneous abortions were a consistent feature of the APS since its original description. They tend to occur during the second and third trimester of pregnancy, at variance with the usual pattern of first trimester pregnancy loss in the general population. The presence of moderate levels of aCL and/or of a LA may confer to a woman a 30% risk of having a miscarriage during her first pregnancy. The risk increases to 70% if a woman had already two miscarriages. There is now some agreement to suggest that the presence of aPLs is a predictor of poor fetal outcome as well as a poor obstetric history (41). Other obstetric associations of aPLs are early onset pre-eclampsia and intrauterine growth retardation. Progressive thrombosis of the placental microvasculature leading to placental insufficiency and infarction underlie these manifestations. However, not all examined placentae disclosed thrombosis or infarction, and other mechanisms may be operating in these patients.

Other vascular manifestations

Kidney involvement may be extraparenchymal or intraparenchymal. In the former case, unilateral or bilateral renal artery occlusion may occur, whereas glomerular thrombosis and a particular form of thrombotic microangiopathy are examples of the latter. Pathological data show re-canalized thrombi without inflammatory infiltrate, at variance with the typical features of lupus nephritis. Nephrovascular hypertension following arterial thrombosis, renal insufficiency and severe proteinuria may contribute to increased morbidity and mortality. Occlusion of mesenteric arteries may cause bowel infarction. Thrombosis of dermal vessels may lead to a variety of skin manifestations including cutaneous necrosis or gangrene, ulcerative lesions, erythematous macules, painful purpura and hemorrhagic bullae. These lesions are characterized by thrombosis of small dermal arterioles, capillaries and venules with no evidence of vasculitis. Interruption of the vascular supply to bones may cause osteonecrosis (42).

Non-thrombotic vascular manifestations

In APS any organ may be affected although it is always possible to attribute the involvement to vascular occlusion. Heart valve lesions are common in APS and strongly associate with the presence of aPL and are progressive in patients with high titre aPL (43). Mitral and aortic valves may become regurgitant and may require replacement. Pulmonary hypertension is less common and may be a primary phenomenon or be secondary to pulmonary microembolism. Movement disorders are linked to the presence of aPLs. A significant association exists between movement disorders (epilepsy and chorea) and aPL and there are cases of transverse myelitis and Guillain-Barré syndrome (42). Skin involvement may appear as livedo reticularis, a blotchy white-purple discoloration due to reduced blood flow through sub-papillary and dermal vessels (42).

Haematological manifestations

Thrombocytopenia is commonly found in the APS, with a prevalence of approximately 20%. Bleeding is not as rare as initially thought and is probably due to an acquired qualitative platelet disorder. Haemolytic anaemia has occurred in SLE patients with aCL generally of the IgM isotype (42).

CHAPTER II

ANTIPHOSPHOLIPID ANTIBODIES AND ATHEROTHROMBOSIS IN SYSTEMIC LUPUS ERYTHEMATOSUS: A UNIFYING 'MEMBRANE STRESS SYNDROME' HYPOTHESIS

Clinical evidence

The survival of patients with SLE is influenced adversely by infection, renal failure and cardiac failure due in part to atherosclerosis and thrombosis (1). With regards to the latter, aPL seem to play a major role, being involved in multiple coagulation defects which lead ultimately to thrombosis. In contrast, the development of atherosclerosis in SLE is poorly understood and the available data are inconclusive as to whether a relation with aPLs exists. Early autopsy studies reported premature coronary atherosclerosis in SLE patients (2,3) and marked atherosclerosis of major cardiac vessels was found in several young SLE patients who died of acute myocardial infarction (4,5). Further studies supported the concept of accelerated coronary atherosclerosis in SLE (6,7). This acceleration was initially attributed to prolonged steroid therapy (6). However, it was subsequently thought that steroids, by suppressing the extravascular manifestations of SLE, allowed patients to live long enough to develop atherosclerosis (8). A more recent controlled autopsy study found coronary vessel involvement to be more premature and prevalent in SLE patients than in age and sex-matched controls and showed that the mean intimal thickening ratio of coronary vessels was significantly higher in SLE patients who had not been treated with steroids compared with those who had been. The authors suggested that a vascular inflammatory process could promote intimal thickening of coronary arteries leading to accelerated atherosclerosis (7). This study however, did not control for the presence of aPLs. On the other hand, an initial relationship found between ischaemic heart disease and aCL (9,10) was subsequently

challenged with the suggestion that the former studies had adopted low cut-off points for the detection of aCL (11, 12). In addition, the low prevalence (4%) of myocardial infarction estimated in a cohort of 300 SLE and non-SLE patients with high levels of aCL is hard to reconcile with the preceding clinical and autopsy observations. Nevertheless, aPLs have been linked to the development of premature re-stenosis of vein autografts in patients undergoing aorto-coronary bypass and premature atherosclerosis may develop in heart transplants following postoperative infection by cytomegalovirus, a virus able to stimulate the appearance of aPL (16). The relation between aPLs and coronary heart disease thus remains to be clarified. Better established is the association between heart valve involvement and aPLs. Thickening of heart valve leaflets leading to varying degrees of valve dysfunction is often found in SLE patients with aPLs, and to a lesser extent, in patients with the PAPS (17-19). Atherosclerosis may also affect cerebral vessels. Several series reported stenosis of intra and extra cerebral arteries in patients who suffered ischemic stroke associated with aPL (20,21) and an autopsy study found arteriolar thickening of cerebral vessels in SLE patients with aPL (22). Despite the reports of an association between aPLs and ischemic stroke (23-25), a large prospective case-control study in men identified aCL (at a titer > 33 GPL units) as a risk factor for venous thrombosis and pulmonary embolism but not for ischaemic stroke (26) and a subsequent study could not find an association between aCL and stroke among survivors of myocardial infarction (27). Therefore, even the relationship between aCL, cerebral thrombosis and atherosclerosis remains equivocal. Data regarding atherosclerotic involvement of other vessels are limited. A few cases of premature non-thrombotic occlusions of lower limb arteries 28 and of renal artery stenosis (29-31) have been described. In some patients, histological findings of the biopsied vessels showed myointimal proliferation with fibrosis, a feature resembling endothelial injury types II and III of accelerated atherosclerosis (32).

Taken together, however, the autopsy studies and the clinical reports suggest that premature atherosclerosis is a feature of SLE.

Lipids, atherosclerosis and thrombosis

Abnormalities of the lipid profile, increased levels of triglycerides (TG) and of very-low density lipoproteins (VLDL)-cholesterol and reduced levels of high density lipoproteins (HDL)-cholesterol, were found in both young and adult untreated SLE patients (33). Steroid treatment may further increase plasma levels of triglycerides and of VLDL-cholesterol, while restoring HDL-cholesterol to normal levels (34). Other studies showed that steroids increased low density lipoprotein (LDL) cholesterol and its major apoprotein-B (Apo-B) (35). The suggestion that an atherogenic lipid profile coupled to the presence of aCL might contribute to the pathogenesis of vascular disease in SLE (36) was confirmed by a study showing that aCL positive SLE patients had lower total cholesterol, HDL-cholesterol and increased Apo-B levels than aCL negative SLE patients (37). The majority of the data point to increased levels of LDL in SLE. It is known that the atherogenicity of LDL may be increased by lipid peroxidation, a process by which free radicals (FR) and reactive oxygen species (ROS) produced in vivo, exert an 'oxidative damage' on lipids, whether isolated or incorporated in polyunsaturated fatty acids converting them into lipid peroxides (38). Oxidised-LDL (ox-LDL) favour monocyte and leucocyte endothelial interactions, recruiting these cells on the arterial wall hence promoting the atherosclerotic process (39,40). Moreover, ox-LDL is more immunogenic than its native form eliciting specific autoantibodies which are currently viewed as independent predictors of carotid atherosclerosis progression (41) and antibodies cross-reacting with ox-LDL and cardiolipin have been found in patients with SLE (42). To complete the atherogenicity of the lipid profile, abnormally high levels of lipoprotein(a) (Lp(a)) have been found in patients with SLE, regardless of previous steroid treatment (43). This apolipoprotein, which shares some structural homology with plasminogen, competes

with the latter and tissue plasminogen activator (tPA) for fibrin binding (45), inhibits fibrinogen uptake and degradation by mononuclear cells (46), modulates the expression of plasminogen activator inhibitor (PAI) in endothelial cells (47) and stimulates smooth muscle cell proliferation (48). Epidemiological studies have ranked Lp(a) as an independent marker of cardiovascular and cerebrovascular disease (49).

Haemostatic variables, atherosclerosis and thrombosis

Multiple mechanisms contribute to the thrombotic tendency of patients with aPLs. An impaired fibrinolytic potential has been reported, often with increased levels of PAI (51,52) but these changes are not always correlated with the presence of aPLs or with the occurrence of thrombosis (53) and were also found in SLE patients without aPLs (54) as well as in normal subjects where hypofibrinolysis represents a predisposing factor for recurrent deep vein thrombosis and/or pulmonary embolisms. However, local or systemic increases in PAI levels are implicated in the development of atherosclerosis (56). Dysfunction of the anticoagulant functions of protein C and protein S (57,58) as well as reduced levels of protein S were found in some patients with aPLs but their relationship with thrombosis could not always be proven (60). Autoantibodies reacting with protein C were detected in a cohort of SLE patients but did not relate to the dysfunctional protein C found in some of the patients nor to a history of thrombosis (62). Impairment of antithrombin function has also been reported (63). It should be remembered that congenital deficiencies of natural anticoagulants predispose to the occurrence of venous thrombosis but rarely of arterial thrombosis. What is the explanation for the latter tendency? Several investigators have explored the role of platelet and vessel wall derived prostaglandins. Their results are conflicting: in vitro, aPLs added to cultured endothelial cells were found to inhibit prostacyclin (PGI₂) release in some studies (64-66) but not in others (67,68). Likewise, sera from patients with SLE and aPLs enhanced the generation of thromboxane-B₂ (TxB₂) from platelets stimulated with collagen or

arachidonic acid but LA had an opposite effect on thrombin stimulated platelets (69). In vivo, urinary excretion of TxB2 metabolites was increased in patients with aCL (70) and LA (71), although no correlation could be found between aCL titres and TxB2 production but, importantly, TxB2 generation was reported to be a persistent phenomenon, supporting the parallel persistence of a hypercoagulable state. In keeping with this, and evidence against the suggestion that the presence of aCL may represent an epiphenomenon of past thrombotic events (72) increased thrombin generation was detected both in thrombotic and non-thrombotic SLE patients with aPL (73), substantiating the presence of an ongoing prothrombotic state. Other studies found increased levels of von Willebrand factor (vWF) in thrombotic and non-thrombotic patients with SLE (74-76) and elevated plasma levels of fibrinogen (FNG) and vWF correlated to the number of occlusive events in patients with aPLs (77). The in vitro observation that vWF factor release from endothelial cells incubated with IgG purified from thrombotic APS patients was increased in respect to IgG from non-thrombotic APS and from controls (78), supports a potential role for vWF in the thrombotic tendency of the APS. Epidemiological studies indicate that high plasma levels of vWF and FNG are risk factors for atherosclerosis and thrombosis (79,80)

The “membrane stress syndrome”

In the mid-1960s it was found that immunologic injury combined with a lipid-rich diet caused the development of atherosclerosis in a rabbit model (81). In the late seventies some non-specific and specific haematological abnormalities appearing in the course of acute and chronic disorders, such as hyperviscosity, reduced fibrinolytic activity and raised levels of fibrinogen and factor VIII (82), were grouped into the definition 'haematological stress syndrome' (83). The mechanisms illustrated in the present overview enclose and extend the former concepts. Given that aPL do not recognise a definite structure in cell membranes, it is possible that aPL may simply induce fluidity changes in membranes that nevertheless trigger

cytoplasmic signalling. Accordingly, the term 'membrane stress syndrome' is proposed to unify the concepts of oxidation, inflammation, autoimmunity, thrombosis and atherosclerosis.

The hypothesis

Analysis of the lipid profile in SLE patients supports the concept of premature coronary and cerebral atherosclerosis in lupus, although prospective studies did not find a relationship between aCL and acute cerebrovascular and cardiovascular events (11, 12, 26). Rather, elevated levels of aCL were identified as a risk factor for venous thrombosis

This is in agreement with the observation that most of the coagulation abnormalities involved in the pathogenesis of APS exposed patients to the risk of venous thrombosis. However, the development of venous and arterial occlusions shares the involvement of the vascular endothelium which due to its location is the prime target for oxidant-mediated injury. Oxidative and peroxidative processes involved in the genesis of atherosclerosis do occur in SLE (85-88). A particular metabolite of lipid peroxidation, 4-hydroxyonenal, activates phospholipases C (PLC) and D (PLD) in vascular endothelial cells (9,90). These enzymes are able to divert the hydrolysis of phosphatidylcholine (PC), the major phospholipid of the outer membrane layer, from the production of PGI₂ to that of platelet activating factor (PAF), a potent platelet agonist (91). Therefore PLC and PLD seem to control the synthetic ratio of PGI₂ to PAF synthesis in the presence of oxidant stress. This is yet another mechanism, probably independent of aPLs, by which the anticoagulant endothelial lining may acquire thrombogenic properties. It may explain the reduced in vivo urinary elimination of PGI₂ metabolites (90, 91) and the discrepancies observed between the production of PGI₂ in vivo and in vitro, where oxidative damage does not occur. The same studies reported increased urinary excretion of 2,3-dinor-TxB₂, a platelet metabolite generated through the β -oxidation pathway (92), a finding which again reveals ongoing oxidative stress in these patients, since ox-LDL may induce platelet activation in vivo (93). In this setting, the synthesis of PGI₂

seems dependent on the availability of PC and PC appears crucial to membrane integrity and function, as its deficiency or peroxidation may predispose to cancer and infection (94,95). In this respect, it is of interest that an *in vivo* study has shown that soybean PC supplementation together with soybean fats may enhance phagocytosis and killing activities of polymorphonuclear cells (94). It is likely that dietary restriction, immobilization by specific antibodies or lipid peroxidation may reduce the availability of PC. However, the oxidative state can be counteracted by specific antioxidant drugs. In homozygous homocystinuria, an inborn error of cysteine metabolism, characterised by premature atherosclerosis, recurrent arterial and venous thrombosis (96), high levels of thiol oxidants are produced that cause endothelial cell injury (97) and the urinary excretion of 2,3-dinor-TxB₂, the β -oxidation pathway catabolite was found to be increased (98) as in the antiphospholipid syndrome. The oral administration of probucol, a drug known for its cholesterol lowering effects, but which also inhibits LDL peroxidation (99), reduced the urinary excretion of 2,3-dinor-TXB₂ (98). Lipid peroxidation may influence surface- dependent haemostasis by externalising from membrane bilayers phosphatidylethanolamine and phosphatidylserine (100), two phospholipids deeply implicated in the coagulopathy of the APS (101, 102). Membrane disruption may enhance reactivity of aPL to erythrocytes, neutrophils and platelets (103) with its physiopathological consequences (72). In addition, oxidative damage may also break through immunological tolerance and enhance autoantibody production (104).

Future directions

Information emerging from studies on the consequences of oxidative damage should open new directions to follow in the pathogenesis of the APS and explain some apparent contradictions. Oxidative stress takes place in the arterial system where primed neutrophils may discharge their oxidative content (105). A vein autografted in the arterial side would be exposed to an oxidative injury not present in the venous system: since the phospholipid

composition of endothelial cells varies according to the arterial or venous location (106), lipid peroxidation might favour premature occlusion of the graft for deficient release of PGI₂ and increased vWF release (107) and promote the endothelial expression of neophospholipid antigens and corresponding aPL. Oxidative damage of endothelial cells might enhance their production of IL-6 which stimulates hepatic synthesis of FNG (108). Moreover, oxidative damage impairs the physiological properties of some coagulative proteins: the heparin binding site of AT is modified (109), plasmin loses its catalytic potential on fibrin/fibrinogen (110), FNG may coagulate in the absence of thrombin (111), PAI can no longer inactivate t-PA (112) and ox-LDL may reduce protein C activation on endothelial cells (113). In the same fashion, oxidative stress may disturb other surface dependent coagulant and anticoagulant pathways. Overall, these in vitro findings would favour a hypercoagulable state if operating in vivo, adding to the thrombogenic potential of aPL and further challenging our preventive options. In this respect, aspirin inhibits platelet but not neutrophil cyclooxygenase, therefore cannot totally block TxA production (114). Aspirin treated platelets may synthesise vasoconstrictor leukotrienes from neutrophil precursors even when they can no longer generate TxA₂ (115). Moreover, platelet aggregation induced by shear forces and mediated by vWF, is an aspirin-resistant process (116). Can these mechanisms explain some aspirin failures in preventing arterial thrombosis in the antiphospholipid syndrome (117,118)? Should we consider antioxidant administration combined with antiplatelet or anticoagulant therapy? Thrombosis may recur also after oral anticoagulation (118,119) and although this may depend on the level of anticoagulation achieved (120) some of the presented coagulation anomalies could be involved. The extent of coronary atherosclerosis is affected by steroid treatment in patients with SLE (7). Does this imply that steroids may affect oxidation processes? Specific studies in the field of membrane and protein oxidation/peroxidation with respect to antiphospholipid antibodies may help elucidate the pathogenesis of premature thrombosis and

atherosclerosis in the 'membrane stress syndrome' and may suggest new mechanisms amenable to therapeutic intervention.

CHAPTER III

METHODS

Blood sampling.

Blood samples were almost always obtained between 8.00 and 9.00 a.m. after an overnight fast and a resting period of 20 minutes. Blood samples for clotting assays were collected by clean venepuncture of an antecubital vein in plastic tubes containing 1/10 volume of 0.129 trisodium citrate. Blood samples for basal PAI and for pre and post occlusion tPA (after 10 minutes of application of the cuff of a sphygmomanometer midway between the systolic and diastolic pressure of the patient) were drawn in similar plastic tubes containing 1/10 volume of 0.129 M trisodium citrate to which theophylline 0.9 mM and prostaglandin E1 1 µg/ml were added to minimize platelet activation.

After centrifuging at 2000 x g for 10 min at 4°C and aliquots of platelet poor plasma (PPP) were stored at -70°C for all determinations, the residual being spun twice at 10000 x g for 5 min at 4°C to obtain platelet free plasma (PFP). Control pooled PFP was obtained from 54 healthy hospital personnel (30F, 24M, mean age 32±18 yrs). This was aliquoted and frozen at -70°C. Blood samples for serum preparation was collected into glass tubes, allowed to clot for 2 hours at room temperature and spun at 1000 x g for 10 minutes. They were stored as plasma samples.

Antiphospholipid antibody assays

PFP for LA assays was processed immediately. LA was screened by 1) activated partial thromboplastin time (aPTT) using rabbit phospholipid-kaolin (Diamed, Switzerland) 2) Kaolin clotting time (KCT) according to the method of Exner (11), 3) dilute Russell's venom viper time (DRVVT) after the method of Thiagarajan (1). A clotting time ratio between sample and control plasma >1.2 for the aPTT and >1.18 for the DRVVT and >1.3 for the KCT

indicated an abnormal result. In any of the assays, a clotting time of a 1:1 mixture of sample and pooled control plasma greater than that of the control plasma alone indicated the presence of an inhibitor whose lupus nature was confirmed by the platelet neutralisation procedure (PNP) according to the original method of Triplett (2) and by high phospholipid concentrations according to Rosove (3). Serum IgG aCL were measured by a commercially available ELISA assay (Melisa system, Byk Goulden, Italy). A normal range was established using the same 54 healthy hospital personnel as stated above, with the cutoff of 5GPL U/ml being 5 standard errors above the geometrical mean (4.1; SEM:0.2; 95% CI 3.7 to 4.5). Titres between 5-20 GPL were considered low positive, between 21-80 GPL medium positive and over 80 GPL high positive. IgG β_2 GPI were measured by an immunoassay from Corgenix, Westminster, USA. A cut-off for positivity was established in 120 healthy controls at 20 units. Inter and intra-assay coefficient of variability were 4.1% and 3% respectively.

ELISA for oxLDL- β_2 GPI complexes

Capture anti- β_2 GPI mAb, WB-CAL-1, was adsorbed on a microtitre plate (Immulon 2HB, Dynex Technologies, Inc., Chantilly, VA, USA) by incubating at 8 μ g/ml (dissolved in Hepes buffer, 50 μ l/well) at 4°C overnight. The plate was blocked with 1% skim milk for 1 h. Serum samples (100-fold diluted) were added to the wells (100 μ l/well) and incubated for 2 h. The wells were subsequently incubated with biotinyl-anti-ApoB-100 antibody (1D2) for 1 h and HRP-labelled avidin for 30 min. Colour was developed with o-phenylenediamine and H₂O₂. The reaction was terminated by 2N sulphuric acid, and the OD at 490 nm was measured. Between each step, extensive washing was performed using Hepes buffer containing 0.05% Tween-20. The mean OD of blank wells corrected the raw OD of samples in individual assays. When 1.0 U/ml was adjusted to 3 S.D. above the mean of serum samples from 50 normal subjects, 1.0U/ml of the oxLDL β_2 GPI complex was equilibrated to 4.5 μ g/ml of ApoB equivalent. A sample was considered positive when its reactivity was >1.0 U/ml.

ELISA for CRP-oxLDL- β_2 GPI complexes

Capture anti- β_2 GPI mAb, WB-CAL-1, was adsorbed onto microtitre plates (Immulon 2HB; Thermo Labsystems) by incubating at 8 μ g/ml (dissolved in Hepes buffer, 50 μ l/well) at 4°C overnight. After blocking with 10 mM Tris, 150 mM NaCl, 1.25 mM CaCl₂, pH7.4, (Tris buffer) containing 0.5% BSA, samples diluted 1:100 with Tris buffer containing 0.2% BSA were added to each well and incubated for 2 h. The wells were subsequently incubated with HRP-labelled anti-CRP antibodies for 1 h. Extensive washing between steps was performed with Tris buffer containing 0.05% Tween-20. Further steps were performed as described above for oxLDL- β_2 GPI complexes.

Plasma fibrinogen and thrombin time

Plasma fibrinogen was measured by the a clotting assay (Mascia Brunelli, Italy) according to Clauss (4) with a CV of 4.2 %. Thrombin times (Bovine thrombin, Boehringer Mannheim, Germany) were performed in duplicate at the same time of the fibrinogen assays.

Plasma fibronectin

Plasma fibronectin was measured by nephelometry (Boehringer Mannheim, Germany).

Von Willebrand factor

Plasma vWF antigen was assayed by an Elisa method (Boehringer Mannheim, Germany). Inter and intra-assay CV for vWF were 3.2% and 4.4% respectively. A normality range between 60 and 150 IU/dl for vWF was determined in 54 healthy subjects.

Natural anticoagulants

Antithrombin and protein C were measured by amidolytic and immunoassays (Behring, Marburg, France) whereas total protein S by immunoassay only (Elisa, Diagnostica Stago, Asnieres, France). Free protein S was measured by a double monoclonal ELISA (Diagnostica Stago, Asnieres, France). Pooled normal plasma from 65 normal donors served as control plasma for PS. Results are expressed as percent of normal.

Markers of coagulation activation

Plasma levels of F1+2, TAT, markers of thrombin generation and D-Dimer were measured by ELISA (Enzygnost TAT and Enzygnost F1+2, Behring Corp., Marburg, Germany and Innatest D-Dimer Byk-Sangtec Diagnostica, Dietzenbach, Germany).

Factor XIII

Factor XIII was measured by a photometric assay (Behring, Germany). The assay is based on the activation of FXIII present in a sample by exogenous thrombin: activated FXIII links a specific peptide substrate with glycine ethyl ester that releases ammonia in the reaction with NADH to yield NAD. The variable measured is the decrease in NADH monitored at 340 nm. A reference range was derived from pooled normal plasma of 30 healthy blood donors. Results are expressed as percentage from the reference curve established using serial dilutions of pooled plasma. The interassay CV (n=15) was 3.6%.

Markers of fibrinolysis

TPA antigen and PAI antigen (as a surrogate of PAI activity) were measured by ELISA (Innotest D-Dimer, Innatest t-PA, Innatest PAI, Byk-Sangtec Diagnostica, Dietzenbach, Germany). Post occlusion values of tPA were corrected for haemo-concentration according to the formula $F = Ht1 (1 - 0.9 \times Ht2) / Ht2 (1 - 0.9 \times Ht1)$. All clotting assays were performed on a KC4 Amelung coagulometer (Amelung, Austria) whereas all immunoassays were read on a standard microplate reader.

Vasoactive molecules and oxidative stress markers

Endothelin-1 was measured by Elisa (R&D Systems, Oxon, UK) following extraction from 1 ml of plasma samples using C18 columns (Nichols Institute Diagnostics, San Juan Capistrano, CA) according to the manufacturer instructions. Urinary 11-dehydro-thromboxane B2 was extracted then measured by a competitive EIA according to the

manufacturer instructions (Cayman Chemical, MI, USA). F2-isoprostanes were measured by gas chromatography followed by mass-spectrometry (5).

Inflammatory markers

High sensitivity C-reactive protein (Biosupply Ltd, Bradford, UK) and serum amyloid A (Europa Bioproducts, Ely, UK) were measured by immunoassay.

Immune activation markers

Neopterin (Biosupply Ltd, Bradford, UK) and soluble CD14 (R&D Systems, Abingdon, UK) were measured by immunoassay.

Nitric oxide metabolites: nitrate, nitrite and nitrotyrosine

Nitrate (NO_3^-) and nitrite (NO_2^-) were determined using the Griess reaction. Serum was diluted 1:4 with PBS (pH 7.4), and 200 μl of this solution was ultrafiltered by centrifugation at 10 000 g for 1 h, using 10 kDa molecular weight filters (Ultrafree-MC; Millipore). Only clear and colourless filtrates were tested. The assay was performed in standard flat-bottomed 96-well polystyrene microtitre plates containing 50 μl /well of standard or sample. The assay was blanked against PBS. To each well were added 4 μl of nitrate reductase and 10 μl NADPH giving final concentrations of 6.3 U/l and 550 $\mu\text{mol/l}$ respectively. Plates were incubated at room temperature for 2 hours. NO concentration was then determined by the addition of 65 μl of Griess reagent 1 and 2 to each well except to blanks, and after 10 min incubation at room temperature the absorbance was read at 540 nm. Nitrotyrosine was measured by enzyme linked immune assays (HyCult Biotechnology, Uden, Netherlands).

Other

Cholesterol, HDL, LDL, ApoAI, ApoB and creatinine were laboratory grade measurements.

Gene polymorphisms

Factor V A506→G, MTHFR C677→T and PT20210 mutations were determined by polymerase chain reaction.

Imaging

Deep vein thrombosis was diagnosed by Doppler ultrasound, pulmonary embolism by perfusional scintiscanning or computed tomography angiography; ischaemic stroke by CT scan and/or MRI; myocardial infarction by ECG and serial changes in cardiac enzymes; other arterial occlusions were diagnosed by arteriography according to the occlusion site.

Measurement of intima media of carotid arteries

High resolution ultrasound of the carotid arteries was performed by an Aloka 2000 sonograph (Aloka Co Ltd, Tokyo, Japan) equipped with a 5–10 MHz linear transducer. The image acquisition followed the Atherosclerosis Risk in Communities Study protocol [12]. Transverse and longitudinal views of the far wall of the right and left common carotid (CC) arteries (10 mm distal to the carotid bifurcation), right and left carotid bifurcation (B) and internal carotid (IC) arteries were taken with the patient in the supine position with their neck slightly extended. The means of three measurements taken 1 cm apart at the three different sites were computed for each region. For the purpose of statistical analysis, right and left measurements were averaged. A plaque was defined as a focal thickening greater than 1.2 mm. Intra-reader reproducibility was assessed in two ways. First, the same carotid measurement was performed twice on 20 individuals yielding a coefficient of agreement of almost 97%. Second, the same IMT measurements were performed six times on one individual over a 2-month period, yielding a coefficient of variability between 4–5% according to carotid segment under study.

CHAPTER IV

ANTIPHOSPHOLIPID ANTIBODIES, COAGULATION, FIBRINOLYSIS AND THROMBOSIS

INTRODUCTION

Ischaemic stroke (IS) and myocardial infarction (MI) represent the major vascular complications of atherosclerosis and are the leading causes of morbidity and mortality in the western countries: at a reduced scale they represent also the leading cause of morbidity and mortality in the APS. The coagulation and fibrinolytic systems are deeply involved in the development of thrombosis and atherosclerosis because they determine the rate of fibrin and D-dimer that is turned over on a daily basis; their activity is finely tuned so that very minor amounts of fibrin generated prevent bleeding from vessel trauma, but at maximal coagulation activation excess fibrin formation may generate an embolic or thrombotic occlusion. Over a lifetime different rates of fibrin turnover between these two extremes, dictated by different plasma concentrations and activities of several coagulation and fibrinolytic proteins including FNG, vWF and PAI may account for the development of atherosclerosis.

Haemostatic variables and atherothrombosis

FNG is the most abundant plasma coagulation factor and behaves as an acute phase reactant: it is synthesised in the liver under the control of three different genes coding for three non identical polypeptide chains (α , β and γ) linked together by disulphide bonds, that allow the assembly of FNG in two symmetrical halves. Under the action of thrombin FNG polymerises into fibrin that is covalently cross-linked by factor XIII. FNG is present in fibrous and atherosclerotic plaques, its plasma concentration is a major determinant of plasma viscosity, of platelet aggregation and of endothelial cell injury, factors that are central to thrombosis and atherosclerosis. The Northwick Park Heart Study showed that one standard

deviation elevation in plasma FNG was associated with an 84% increased risk of an episode of ischaemic heart disease within 5 years (1). Epidemiological evidence has clearly linked high plasma FNG concentrations with the risk of cardiovascular and cerebrovascular disease (reviewed in 2).

vWF is a multimeric glycoprotein circulating in plasma in association with factor VIII, it is synthesized constitutively in endothelial cells (in the Weibel-Palade bodies) and in megakaryocytes (α -granules of platelets) and its main function is to mediate platelet adhesion between themselves and to endothelial cells. Plasma vWF concentration increases after endothelial activation or injury favouring thus platelet activation: epidemiological studies have ranked vWF as a strong predictor of IS, MI and deep vein thrombosis (DVT) (3).

Tissue type plasminogen activator (t-PA) and inhibitors such as PAI and α 2-antiplasmin maintain the balance of the intravascular fibrinolytic activity. Mainly of endothelial cell origin t-PA and PAI belong to the serine protease inhibitor super-family and they are necessary to prevent excessive fibrin formation and deposition. Population based surveys have highlighted their association with IS, MI and atherosclerosis (4-6). Factor XIII (FXIII) is a transglutaminase of endothelial origin that cross-links fibrin with itself and with the endothelial matrix and is implicated in thrombosis and atherosclerosis (7).

Haemostatic variables and thrombosis in PAPS

The association between aPL and thrombosis is widely recognized regardless of the underlying disorders associated with the presence and persistence of aPL (8). The pathogenesis of vascular occlusion in APS is multi factorial: depressed fibrinolysis and interference with the natural anticoagulant systems leads to excess thrombin generation that may explain the susceptibility to venous thrombosis. In addition, the joint effect of unopposed thrombin generation on thromboxane production via platelet activation and of reduced

prostacyclin released from endothelial may explain the susceptibility to arterial thrombosis (9).

Increased plasma levels of FNG and vWF have been reported in APS (10-12), though their relationship with type of vascular occlusion is poorly known. Persistent thrombin generation has been demonstrated in thrombotic and non thrombotic aPL positive patients with SLE (12) in relation to free protein S (f-PS) deficiency (12) underlining an association between aPL and a prothrombotic state, eventually mediated by low f-PS (12). The effect of aPLs on the fibrinolytic system has been investigated mostly in autoimmune diseases with aPL, with controversial results, probably due to the small size of the cohorts and of the heterogeneity of the patients studied (13-45) and the contributory role of factor XIII to the hypercoagulable state of APS has never been explored.

To test the hypothesis that aPL, coagulation and fibrinolysis markers related to thrombosis subtype in APS, three different studies were carried out: the first assessed the role of FNG and vWF, the second the role of coagulation activation and fibrinolysis markers and the third the possible role of factor XIII.

EXPERIMENTAL DATA

1) ANTIPHOSPHOLIPID ANTIBODIES, FIBRINOGEN, VON WILLEBRAND FACTOR AND THROMBOSIS

This study was carried out on 144 consecutive patients (103F, 41M, mean age 35 ± 14 years) found positive on two separate occasions three months apart. They were evaluated because of a history of thrombosis, for diseases known to be associated with the presence of aPL and/or for coagulation anomalies suggesting the presence of a lupus anticoagulant (LA). To maintain the focus of the study on the prevalence of arterial and venous thrombosis and its relationship with haemostatic variables, women with miscarriages (with or without

thrombosis) or with a obstetric history linked to aPL were excluded as they were likely to act as confounders.

APL was detected in 56 (39%) patients with autoimmune thrombocytopaenic purpura (ATP) (diagnosed according the American Society of Haematology Criteria) (16), in 43 (30%) patients with SLE (diagnosed according the American Rheumatism Association Criteria (17) and in 45 (31%) patients with no underlying disorder (NUD) (Table 1). The methods for diagnosing deep vein thrombosis, pulmonary embolism, ischemic stroke and pulmonary embolism as well as the assays for the detection of plasma FNG, vWF, IgG aCL and LA are described in the methods chapter.

Table 1. Demographics.

	ATP	SLE	NUD
Patients (No)	56	43	45
Female/male	44/12	37/6	22/23
Age (yrs) (mean \pm SD)	37 \pm 14	29 \pm 10	38 \pm 15
Thrombotic patients (No/%)	5 (9)	12 (28)	29 (64)

ATP: autoimmune thrombocytopaenic purpura; SLE: systemic lupus erythematosus; NUD: no underlying disorder.

RESULTS

Prevalence of lupus anticoagulant and IgG aCL

At least one LA test was positive in 125 (86%) patients: aPTT in 51 (40%), KCT in 98 (78%) and DRVVT in 45 (36%). All LA were confirmed by a phospholipid neutralisation method. In particular PNP confirmed 43 (83%) aPTTs and 38 (72%) DRVVTs, whereas high phospholipid concentration confirmed 59 (62%) KCTs. A total of 126 (87%) patients presented with positive levels of IgG aCL, 67 (53%) in the low range, 40 (31%) in the medium range and 19 (15%) in the high range. A concordance between IgG aCL and LA was

found in 109 (75%) patients: 17 (13%) LA positive patients were IgG aCL negative and 18 (14%) IgG aCL positive patients were LA negative.

Prevalence of thrombotic patients according to disease subgroup

A history of single or multiple vascular occlusions was present in 46/144 (32%) of patients (25 females, 21 males). Arterial thrombosis occurred in 12 patients (8%), venous thrombosis in 28 (19%) patients and both events in 6 (4%) patients. The prevalence of patients with a history of thrombosis was higher in the group with no underlying disorder (NUD) 64%, than in the groups with SLE, 28% and ATP 9% ($p<0.0001$). Of the occlusive events ($n=76$), deep vein thrombosis was the most common, 36 (47%) followed by ischaemic stroke 18 (23%) and pulmonary embolism 9 (11%) (Table 2).

Table 2. Details of vascular occlusions

	ATP	SLE	NUD
Venous occlusions			
Lower limb	5	11	20
Upper limb		1	2
Mesenteric			2
Pulmonary embolism			9
Arterial occlusions			
Ischaemic stroke	2	3	13
Myocardial infarction	1		1
Lower limb			3
Mesenteric			1
Splenic			1
Renal			1

ATP: autoimmune thrombocytopaenic purpura; SLE: systemic lupus erythematosus; NUD: no underlying disorder.

Relationship between antiphospholipid assays and thrombosis

Amongst the aPL assays, aPTT and DRVVT correlated to a history of thrombosis ($p=0.01$ and $p<0.0001$ respectively) whereas IgG aCL did not (Table 3A). Considering separately arterial and venous thrombosis DRVVT and aPTT both correlated to a history of venous thrombosis ($p=0.0002$ and $p=0.009$ respectively) but only DRVVT correlated with a history of arterial thrombosis ($p=0.01$) (Table 3B).

Table 3A. Relationship between antiphospholipid assays and any thrombosis.

		Thrombosis		OR	95% CI	p-value
		+	-			
aPTT	+	23	28	2.50	1.21, 5.17	0.01
	-	23	70			
KCT	+	28	70	0.62	0.3, 1.30	0.2
	-	18	28			
DRVVT	+	28	17	7.41	3.36, 16.34	<0.0001
	-	18	81			
IgG aCL	+	44	82	4.29	0.94, 19.53	0.057
	-	2	16			

Table 3B. Relationship between antiphospholipid assays and thrombosis subtype.

		Thrombosis		OR	95% CI	p-value
		Arterial				
		+	-			
aPTT	+	10	10	1.95	0.75, 5.06	0.2
	-	42	82			
DRVVT	+	13	11	3.21	1.32, 7.96	0.01
	-	32	88			
		Venous				
aPTT	+	18	14	2.95	1.31, 6.60	0.01
	-	34	78			
DRVVT	+	20	12	5.51	2.38, 12.76	<0.0001
	-	26	86			

Relationship between IgG aCL and thrombosis

Sixteen patients (24%) in the low IgG aCL range, 14 (35%) in the medium IgG aCL range and 14 (73%) in the high IgG aCL range underwent at least one thrombotic episode. A higher mean IgG aCL was found in patients who suffered thrombosis than in those who did not ($p < 0.0001$, Table 4).

Table 4. Geometrical means of FNG, vWF and IgG aCL according to type and number of thromboses

Any thrombosis	Number of occlusions				p-value#
	0	1	2	3+	
	(n=98)	(n=26)	(n=14)	(n=6)	
FNG mg/dl	270	364	422	505	<0.0001
vWF IU/dl	98	147	143	191	<0.0001
IgG aCL GPL	15	25	42	124	<0.0001
Venous thrombosis	Number of occlusions				p-value#
	0	1	2	3+	
	(n=110)	(n=22)	(n=8)	(n=4)	
FNG mg/dl	284	389	375	387	<0.0001
vWF IU/dl	102	154	140	183	<0.0001
IgG aCL GPL	15	32	53	131	0.0001
Arterial thrombosis	Number of occlusions				p-value#
	0	1	2		
	(n=125)	(n=12)	(n=6)		
FNG mg/dl	289	376	665		<0.0001
vWF IU/dl	108	136	176		0.002
IgG aCL GPL	18	46	32		0.01

p-value for testing mean where no event vs mean where at least one event. All variables analysed on log scale, FNG: fibrinogen; vWF: Von Willebrand factor; aCL: anticardiolipin antibody.

Relationship between von Willebrand factor and thrombosis

Plasma levels of vWF were <60 IU/dl in 17 (12%) patients, between 61 and 150 IU/dl in 95 (66%) patients and >150 IU/dl in 32 (22%) patients. Two patients (12%) at vWF <60 IU/dl, 19 (20%) patients at vWF between 61-150 IU/dl and 25 (78%) patients at vWF >150IU/dl experienced at least one thrombotic episode. Significantly higher plasma levels of vWF were found in thrombotic than non thrombotic patients ($p<0.0001$, Table 4). Mean vWF was significantly higher in aPL positive thrombotic patients than in a control group of aPL negative thrombotic patients with inherited thrombophilia [151 (7.9) vs 121 (4.7), $p<0.0001$].

Relationship between fibrinogen and thrombosis

Plasma levels of FNG were <300 mg/dl in 75 (52%) patients, between 301-400 mg/dl in 40 (28%) patients, between 401-500 mg/dl in 19 (13%) patients and >500 mg/dl in 10 (7%). Seven (9%) patients at the lowest FNG level, 18 (45%) patients at FNG between 301-400 mg/dl, 13 (68%) at FNG between 401-500 mg/dl, and 8 (80%) patients at FNG >500 mg/dl experienced at least one thrombotic episode (Table 4). Mean FNG in aPL positive thrombotic patients was significantly higher than that of aPL negative thrombotic patients [397(18.9) vs 305 (9.7), $p<0.0001$].

Regression analysis of FNG, vWF and IgG aCL with thrombotic events

Two possibilities were considered. 1) That the occurrence of the first event was qualitatively different from the occurrence of subsequent events and that this manifested in differential rates of change in outcome at the first and subsequent events. 2) That the rate of change for the first event was the same as for subsequent events. These hypotheses were tested simultaneously and estimates are presented only for those parameters for which significant evidence was found of non-zero effect. Data were analysed according to the groups presented in Table 5: values of IgG aCL, FNG and vWF in patients with 0,1,2 or 3 thrombotic events were entered in the regression model.

Table 5. Regression analysis of FNG, vWF, and IgG aCL versus numbers of events after adjustment for any confounding effect of age, sex or group (ATP, SLE, NUD) as required

Event type	Variable	Confounders p-value	Mean % increase for 1 st event (95% IC)	p-value	Mean % increase for subsequent events (95% IC)	p-value
All events	FNG	Age 0.0003	35% (21, 50)	0.02	11% (2, 20)	0.0016
	VWF	Age 0.04	50% (31, 72)	0.001		
	IgG aCL	Sex 0.03			54% (24, 90)	0.04
		Group 0.0001				
Venous	FNG	Age 0.0002	27% (12, 43)	0.001		
	VWF	Age 0.004	40% (20, 66)	0.001		
	IgG aCL	Sex 0.02			58% (23, 102)	0.001
		Group 0.0001				
Arterial	FNG	Age 0.0004			37% (34, 51)	0.001
	VWF	Age 0.007			18% (1, 37)	0.002
	IgG aCL	Sex 0.003	64% 8, 192)	0.02		
		Group 0.0001				

ATP: autoimmune thrombocytopaenic purpura; SLE: systemic lupus erythematosus; NUD: no underlying disorder; FNG: fibrinogen; vWF: Von Willebrand factor; aCL: anticardiolipin antibody

In respect of total events FNG rose by an average of 38% (95% CI; 24%, 54%) on occurrence of the first event and by about 10% per event thereafter. By contrast vWF rose by about 54% (95% CI; 34%, 75%) at the first event and was unchanged thereafter whether or not subsequent events occurred, whilst IgG aCL rose by 75% (44%, 112%) per event throughout. When analysed for the number of venous events, FNG rose by 36% (95% CI; 21%, 53%) and vWF by 50% (95% CI; 29%, 75%) at the first venous event and remained unchanged thereafter. With respect to arterial events, FNG rose by 45% (95% CI; 31%, 60%)

per arterial event and vWF by 27% (95% CI; 10%, 47%) per arterial event throughout. IgG aCL rose by 131% (95% CI; 28%, 320%) at first arterial event and remained constant thereafter. After adjustment for confounders (age, sex and group) results were relatively unchanged though actual estimates were somewhat diminished (Table 5).

Relationships amongst IgG aCL, vWF and FNG

Significant correlations were noted between IgG aCL and vWF ($r=0.42$, $p<0.0001$), IgG aCL and FNG ($r=0.23$, $p=0.005$) and between vWF and FNG ($r=0.48$, $p<0.0001$).

Natural anticoagulant measurements

Reduced plasma levels of protein C antigen (55%; NV 70-120%) and total protein S (58%; 70-120%) were found in two patients: both experienced thrombosis. Values were substantially unchanged (53% for protein C and 62% for protein S) of further testing six months later. Family members were unaffected by the defect. Thrombin times and plasma levels of AT were normal in all patients.

2) ANTIPHOSPHOLIPID ANTIBODIES, THROMBIN GENERATION AND FIBRINOLYSIS

This study was carried out on 18 patients with thrombotic PAPS (8M, 10F mean age 37 ± 13), 18 non thrombotic persistent carriers of aPL with no underlying disease (7M, 11F, mean age 38 years) and on 20 hospital personnel (9M, 11 F mean age 36 ± 11) as a healthy control group. To validate thrombin generation markers in the PAPS group we also employed patients with inherited thrombophilia (juvenile ischaemic stroke IS $n=8$, unexplained deep vein thrombosis $n=7$, deep vein thrombosis secondary to protein C deficiency $n=3$) without aPL. Information regarding smoking status, hypertension, alcohol intake, hypertension, menopause and contraceptive use was obtained from all participants (Table 6).

Table 6. Demographics

	CTR	APL	PAPS
Subjects	20	18	18
Sex (F/M)	11/9	11/7	10/8
Age years (mean±SD)	36±11	39±12	37±13
IgG aCL <20 GPL	-	6	4
IgG aCL 20-80 GPL	-	6	4
IgG aCL >80 GPL	-	2	9
LA only	-	4	1
Smokers	1	1	1
Hypertensive	0	1	1
Menopausal	1	1	1

CTR: control; APL: antiphospholipid positive without thrombosis; PAPS: primary antiphospholipid syndrome; aCL: anticardiolipin; LA: lupus anticoagulant.

Antiphospholipid status of patients

None of the subjects in the control group had a positive level of IgG aCL or a positive LA test. In the aPL group (n=18), 4 subjects (22%) had a confirmed LA only, 6 (33%) had low positive IgG aCL, 6 (33%) had medium levels of IgG aCL, and 2 (11%) had high levels IgG aCL. All IgG aCL positive subjects had at least one positive confirmatory LA test. In the PAPS group (n=18), 1 patient (5.5%) had a confirmed LA (negative aCL), 4 (22.2%) had low positive IgG aCL (matched in all cases by a confirmed LA), 4 (27.7%) had medium positive IgG aCL (matched in two cases by a confirmed LA), the remaining 9 (44.4%) had high positive IgG aCL (matched in all cases by confirmed LA). PAPS patients presented higher geometrical mean levels of IgG aCL than non thrombotics (86.6 ± 23 vs 38.7 ± 13 GPL, $p=0.03$). Of the PAPS patients, 6 underwent arterial occlusions (6 F), 3 arterial and venous occlusions (1F) and 9 venous occlusions.

Comparison of coagulation activation markers between patients and controls

In the aPL groups, measurements of F1+2, TAT and D-D made two months apart were highly correlated ($r=0.89$, $r=0.79$ and $r=0.81$ respectively, p always <0.0001). Mean F1+2 and D-D were significantly higher in PAPS and aPL subjects than in controls whereas those of TAT did not differ (Table 7). A weak correlation between mean F1+2 and D-D in the PAPS group was noted ($r=0.46$, $p=0.052$). Mean D-D (207 ± 43 ng/ml) and F1+2 (1.08 ± 0.15 nmol/L) of PAPS patients were similar to those of thrombotic patients for reasons other than aPL (181 ± 24.4 ng/ml and 1.04 ± 0.13 nmol/L respectively).

Table 7. Thrombin generation markers, D-dimer and fibronectin across study groups

	CTR	APL	PAPS	p-value
Subjects (No)	20	18	18	
F1+2 (nM)	0.6 ± 0.05	1.08 ± 0.07	1.08 ± 0.15	0.006
TAT (ng/ml)	3.03 ± 0.43	2.8 ± 0.17	2.5 ± 0.18	NS
D-D (ng/ml)	59.7 ± 4.42	109 ± 14.6	207 ± 43.5	<0.0001
FNT (mg/dl)	30 ± 0.76	28.1 ± 0.91	26 ± 0.89	0.001
f-PS (%)	103 ± 3.15	84 ± 4.9	85.3 ± 3.45	0.0006

CTR: controls; aPL: persistent positive aPL without thrombosis; PAPS; primary thrombotic antiphospholipid syndrome; F1+2: prothrombin fragment 1+2; TAT: thrombin-antithrombin complexes; D-D: D-dimer; FNT: fibronectin; f-PS: free protein S.

However, mean TAT of PAPS patients was significantly lower than that of thrombotic patients for reasons other than aPL (2.5 ± 0.18 vs 4.1 ± 0.32 ng/ml, $p<0.0001$). No further measurements were carried out in thrombotic patients without aPL. The following data refer again to our aPL positive subjects. Mean PS was lower in PAPS and aPL patients than in the control group as well as mean FNT (Table 7). In the PAPS group mean FNT was inversely correlated with mean D-D ($r = -0.61$ $p=0.007$)

Comparison of fibrinolysis markers between patients and controls

Mean basal tPA was higher in PAPS (17.0 ± 0.9 ng/ml, $p < 0.0001$) and aPL subjects (12.2 ± 0.5 ng/ml, $p = 0.0009$) than in controls (8.8 ± 1.08 ng/ml) and correlated to D-D in both groups ($r = 0.74$, $p = 0.0004$ and $r = 0.534$, $p = 0.023$, respectively). Mean PAI was not significantly different. Gender analysed data revealed impaired tPA release in PAPS females (from 16.8 ± 0.7 to 21.3 ± 3.9 ng/ml, NS) but not in PAPS males (from 18.2 ± 2.0 to 33.7 ± 4.9 ng/ml, $p = 0.01$) (Table 8). TPA release was unimpaired in aPL subjects of either sex. Females had higher mean PAI than males in the PAPS and aPL group ($p < 0.0001$ and $p = 0.0002$ respectively) and than control females (Table 8). Having established a reference range (mean ± 2 SD) for PAI values in our 20 control subjects, elevated PAI was present in 22% (4/18, 4F) of aPL subjects and in 33% of PAPS patients (6/18). Deficient tPA release (post values $<$ pre values) was present in 11% (2/18, 1F, 1M) of aPL and in 40% (4/18, all F) of PAPS patients. Altogether, deficient tPA release and elevated PAI were present in 27% (5/18) of aPL subjects (one patient with both defects) but in 55% (10/18) of PAPS patients (4 patients with both defects).

Relationship between free PS, thrombin generation and fibrinolysis defects.

By determining a reference range for f-PS ($102.9 \pm 20\%$, mean ± 2 SD), 50% (9/18) of PAPS and 33% (6/18) of aPL subjects had abnormally low levels of f-PS. In both groups, patients with low f-PS generated more F1+2 than patients with normal f-PS (1.4 ± 0.28 vs 0.7 ± 0.08 nmol/L, $p = 0.01$ and 1.3 ± 0.12 vs 0.9 ± 0.08 nmol/L, $p = 0.02$ respectively). Low f-PS associated with fibrinolysis defects in PAPS (90%, 9/10 vs 12.5%, 1/8, $p < 0.01$) and in aPL patients (100%, 5/5 vs 7.7%, 1/13, $p < 0.005$). In PAPS, low f-PS was found in 4 patients with elevated PAI, in 2 patients with elevated PAI combined with defective tPA release and in 3 patients with defective tPA release. In aPL low f-PS was found in 3 subjects with elevated PAI, in 1

subject with elevated PAI combined with defective tPA release, in 1 subject with defective tPA release and as an isolated finding in 1 case.

Table 8. Gender analysed fibrinolytic markers across study groups.

		CTR	APL	p-value	PAPS	p-value
Subjects (M/F)		9/11	7/11		8/10	
tPA pre (ng/ml)	M	10.8±1.6	12.5±1.1	NS	18.2±2.0	0.01
tPA post (ng/ml)	M	13.8±1.7	19.0±1.7	0.01	33.7±4.9	0.0004
					P=0.01	
tPA pre (ng/ml)	F	6.3±0.7	12.1±0.7	<0.0001	17.0±0.9	<0.0001
tPA post (ng/ml)	F	9.5±0.6	21.4±1.7	0.0001	21.3±3.9	0.0003
					NS	
PAI (ng/ml)	M	35.8±6.0	24.5±3.0	0.007	23.4±2.9	0.003
PAI (ng/ml)	F	30.4±4.0	42.0±2.2	0.02	64.0±8.2	0.002
			p=0.0002		p<0.0001	

CTR: controls; aPL: persistent positive aPL (without thrombosis); PAPS; primary thrombotic antiphospholipid syndrome; tPA: tissue plasminogen activator; PAI: plasminogen activator inhibitor

3) ANTIPHOSPHOLIPID ANTIBODIES, FACTOR XIII AND FIBRINOLYSIS

Patients

The study was carried out on 99 consecutive patients, of whom 29 had thrombotic PAPS (22F, 7M, mean age 35±10 years), 14 (11F, 3M, mean age 34±10 years) were persistent carriers of idiopathic aPL in the absence of any underlying disorder and 24 (16F, 8M, mean age 36±8 years) were patients who suffered thrombosis for inherited thrombophilia (IT) (Factor V Leiden n=12, prothrombin n=10, protein C deficiency n=2) and not aPL. All thrombotic PAPS patients met the Sapporo Revised criteria (1). Twenty two PAPS patients suffered venous events (2 events in 5 patients, 3 events in 1 patient), 4 suffered arterial events (2 events in 3 patients) and 3 suffered events in both arterial and venous districts. In the IT

group, 20 underwent venous thrombosis (5 patients with 2 events) and 4 arterial thromboses. Patients with idiopathic aPL were detected because of the presence of a lupus anticoagulant on routine clotting screen for minor surgical procedures and/or for health checks and clinically never suffered any manifestation of APS. At the time of the study, 25 primary APS patients were on warfarin with an INR between 2.0-3.0 and four had an INR between 3.0-4.0. In the aPL group 3 were on aspirin, whereas all patients in the inherited thrombophilia group were on warfarin within an INR 2.0-3.0. To control for a possible effect of warfarin on FXIIIa in a non-thrombotic group, FXIIIa was also measured in 32 patients (18F, 14M, mean age 49 ± 15 years) taking warfarin for valve replacements (mitral n=16, aortic n=16). At the time of FXIIIa determination patients with mitral valve replacement had an INR between 3.0-4.0 and those with aortic valve replacement had an INR between 2.0-3.0. Healthy hospital personnel (n=28, 18F, 10M, mean age 34 ± 9 years) served as a control group. None of the participants were on lipid lowering agents or drugs with anti-oxidant properties at the time of the study.

RESULTS

Factor XIIIa and other variables across groups.

To assess the persistence of activated FXIII, this was re-measured after three months in 15 primary APS patients yielding a coefficient of correlation $r=0.91$ ($p<0.0001$) and in 15 thrombotic controls yielding a coefficient of correlation $r=0.89$ ($p<0.0001$). The proportion of patients with FXIIIa above the mean+2SD of normal controls was 38% (11/29) in PAPS, 7% (1/14) in aPL, 16.6% (4/24) in IT, 12.5% (4/32) in valvular controls and 3.5% (1/28) in normal controls ($p=0.006$). Because FXIIIa could be influenced by the intake of warfarin (14), FXIIIa was also measured in non- thrombotic valvular controls at different INR intensities, though no differences were detected, as FXIIIa was $106\pm 24\%$ in those with INR 2.0-3.0 (n=16) and $110\pm 22\%$ in those with INR 3.0-4.0 (n=16). Thus the valvular control group

consisted of all 32 patients. Median FXIIIa was increased in PAPS than other groups considered (Figure 1). In addition, PAPS had higher FNG and PAI than the other groups (Table 9).

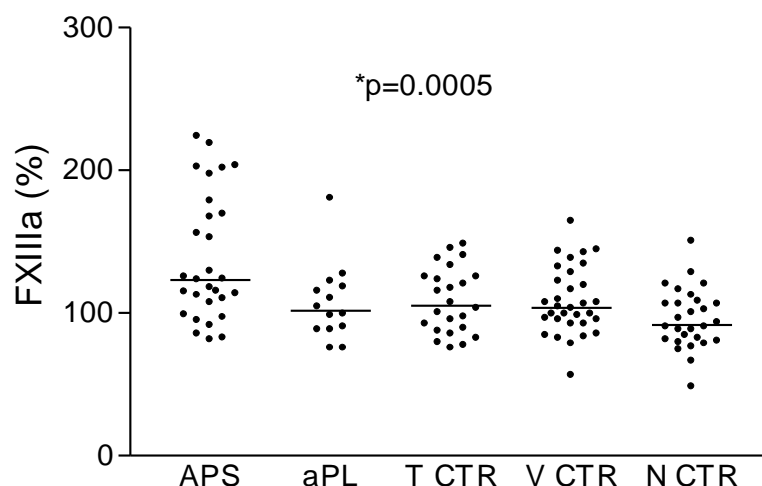


Figure 1. Median levels of factor XIII activity (FXIIIa) in patients with primary thrombotic antiphospholipid syndrome (APS), idiopathic antiphospholipid antibodies (aPL), thrombotic controls (T CTR), valvular controls (V CTR) and normal controls (N CTR). *Analysis of variance (Kruskall-Wallis). APS vs N CTR $p < 0.001$, APS vs aPL $p < 0.05$. (Dunn's Multiple Comparison Test).

Table 9. Antiphospholipid antibodies, FNG and PAI across groups.

	CTR	IT	APL	PAPS	*p-value
IgG aCL (GPL)	6±3.7 (1.2-14)	5±2.6 (1.6-12)	80±87 (16-309)	159±135 (22-573)	<0.0001
IgG β_2 GPI (U)	5.2±2.8 (2.1-12)	4.8±3.5 (1.3-16)	119±61 (40-216)	172±46 76-226	<0.0001
FNG (mg/dl)	251±56 (185-364)	279±62 (198-405)	283±64 (176-364)	342±53 (209-464)	<0.0001
PAI (ng/ml)	30±9 (12-44)	38±15 (20-80)	35±19 (12-76)	43±18* (14-87)	0.06

PAPS: thrombotic primary antiphospholipid syndrome; aPL: idiopathic carriers of antiphospholipid antibodies; IT: inherited thrombophilia; CTR: normal controls; IgG aCL: anticardiolipin antibody; FNG: fibrinogen; PAI: plasminogen activator inhibitor. *Kruskall Wallis. APS vs N CTR $p < 0.05$ (Dunn's post hoc).

Factor XIII in antiphospholipid positive groups

Although FXIII was not significantly different between patients with arterial and venous occlusions (145 ± 54 vs 125 ± 38 %) in PAPS, patients with multiple events ($n=12$) showed a higher FXIIIa than those who had suffered one event only (158 ± 45 vs $118 \pm 38\%$, $p=0.02$). FXIIIa correlated with IgG aCL both in the APS and aPL group (Figure 2A and 2B). Similar results were observed between FXIII and IgG $\alpha\beta_2$ GPI (Figure 2C and 2D).

Furthermore, in PAPS patients FXIIIa correlated to PAI ($r=0.54$, $p=0.003$) and FNG ($r=0.51$, $p=0.005$). The correlation between FXIIIa and PAI was almost reflected in aPL patients ($r=0.51$, $p=0.06$).

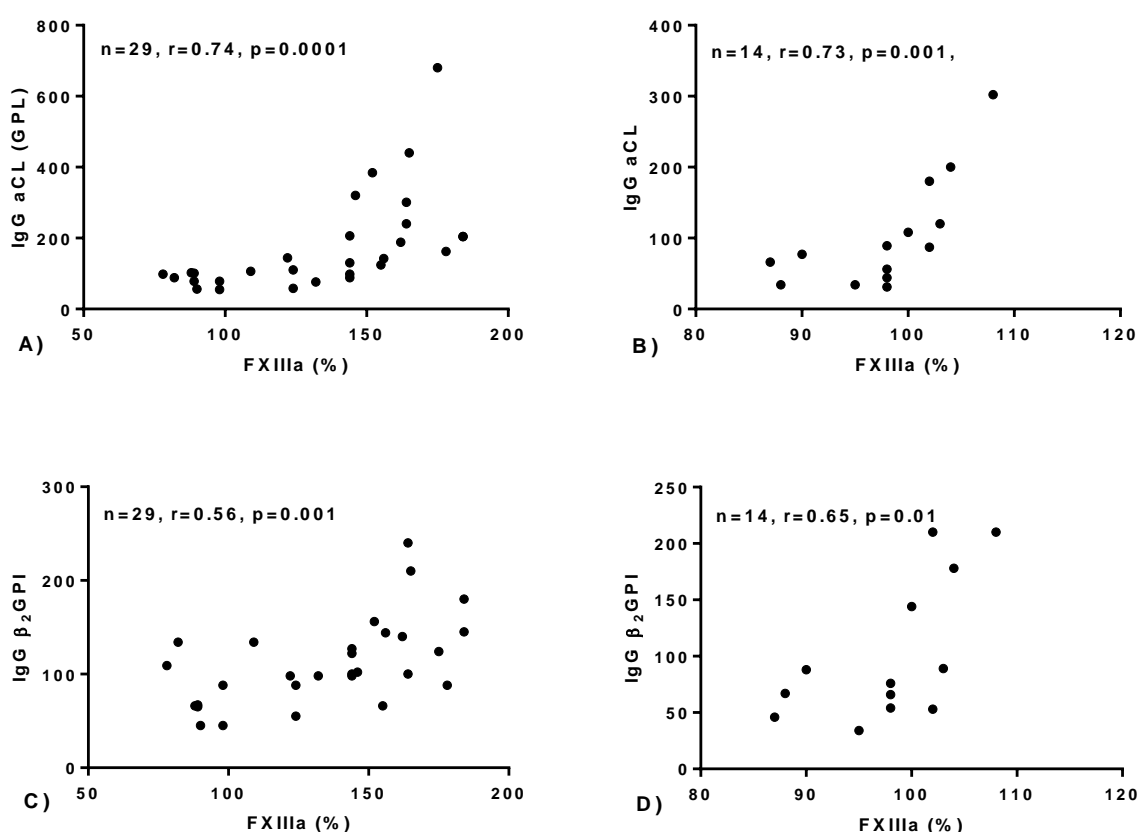


Figure 2. Spearman rank correlations between: factor XIII activity (FXIIIa) and IgG anticardiolipin antibodies (aCL) in primary antiphospholipid syndrome (A) and in idiopathic carriers of antiphospholipid antibodies (B); FXIIIa and IgG anti- β_2 -glycoprotein-I (β_2 gGPI) in primary antiphospholipid syndrome (C) and aPL (D).

Factor XIII in control groups

In the IT group, FXIIIa positively correlated with FNG ($r=0.53$, $p=0.007$) and PAI ($r=0.04$, $p=0.05$). None of these correlations were noted in the normal control group.

DISCUSSION

Thrombotic role of vWF and FNG

Thrombosis represents the most ominous manifestation of the APS, whether occurring in SLE, non-SLE disorders or in otherwise healthy people (8, 18). In the present study patients with a history of thrombosis whether arterial or venous, were more frequent in the group with no underlying disorders (64%) than in SLE (28%) and ATP (9%) groups. These findings are in agreement with previous studies showing a thrombosis prevalence of 54% in patients with idiopathic aPLs (18) and with those of a major review which calculated a thrombosis prevalence of 25% and 28% in LA positive and IgG aCL positive SLE patients (8). LA was found to identify a subset of aPL patients more prone to develop thrombosis both in SLE (12) and non SLE disorders (19, 20). Our findings reflect this tendency since DRVVT and aPTT strongly correlated to a history of thrombosis (Table). However, when analysed by thrombosis subtype, only DRVVT correlated to a history of arterial thrombosis. Whilst IgG aCL strongly associated with the number of venous occlusions, its weak correlation with the number of arterial thromboses became non-significant after correction for confounders. A relationship between high aCL IgG titre and thrombosis was established in both primary and secondary APS (21-25) although there are still controversial issues with regards to thrombosis subtype. One study did not find a different prevalence of venous thrombosis between IgG aCL positive and negative patients (26), a nested case-control study in men revealed high IgG aCL titres to be a risk factor for venous but not arterial thrombosis (27) and IgG aCL did not correlate with ischaemic stroke in young survivors of myocardial infarction (28). In line with these reports

our data support a relationship between IgG aCL and venous thrombosis. Nevertheless arterial thrombosis is a consistent feature of the APS. Owing to the role of FNG and vWF as an independent risk factors for arterial thrombosis and atherogenesis (29, 30) we hypothesised that these two adhesive proteins could be involved in the thrombotic tendency of APS.

Both proteins strongly correlated to the number of vascular occlusions, the differential relationships of FNG with arterial thrombosis and of vWF with venous thrombosis being favoured (Table). Also, mean plasma levels of FNG and vWF were significantly higher in aPL positive thrombotics than in aPL negative thrombotic controls (Table), indicating that aPL might account for the difference. The in vivo correlation found between IgG and vWF in the present study is supported by in vitro data showing that endothelial cells exposed to IgG purified from APS patients release higher amounts of vWF than when exposed to IgG purified from controls (31). The correlation found between FNG and vWF suggests a pivotal role for FNG, being able as fibrin to induce endothelial changes and vWF release (32) and behaving as an acute phase reactant to increase its levels in response to cytokines of vascular origin (33).

Impairment of fibrinolysis leading to heightened fibrin turnover

The occurrence of thrombosis must be underlined by thrombin generation, and a known relationship exists between aPLs and increased thrombin generation in patients with SLE particularly in SLE patients with aPL related f-PS deficiency (12), a phenomenon demonstrated also in non-SLE patients (15, 34). Conversely, data regarding the fibrinolytic system in the APS are still controversial, studies revealing normal (12, 13) or abnormal (14, 15, 35) fibrinolytic patterns. These issues were re-evaluated by measuring several coagulation and fibrinolytic parameters in a rare group of asymptomatic carriers of aPL (that is, subjects with no other anomaly than the persistence of aPL) and in thrombotic patients with PAPS. The conversion of prothrombin to thrombin releases the inactive prothrombin fragment F1+2

and newly generated thrombin is neutralised by the antithrombin-heparan sulphate (AT-HS) inhibitory system followed by formation of an inactive TAT complex. In this study, elevated F1+2 levels were present not only in PAPS patients but also in asymptomatic aPL subjects, and within both groups, levels were significantly higher in subjects with low f-PS, implying a higher rate of thrombin generation in individuals with acquired f-PS deficiency. However, mean TAT levels were neither different between patients and controls, nor did they match the elevated levels of F1+2 in the patient groups. These discrepancies, together with the higher mean TAT level in thrombotic patients without aPLs compared to that in PAPS patients, indirectly suggest a defective function of the AT-HS in subjects with aPLs, in keeping with the notion that binding of aPL to heparin-sulphate can prevent formation of TAT complexes (36) and in agreement with a report showing higher levels of TAT complexes in SLE patients without aPL compared to SLE patients with aPL (37). Thrombin generation leads to FNG polymerisation and subsequent degradation by plasmin with release of the fibrin cleavage product D-D, expression of fibrin turnover. Mean D-D level was elevated in thrombotic and to a lesser extent in non thrombotic patients, further denoting coagulation activation and plasmin lysis in asymptomatic carriers of aPLs. Unexpectedly, mean FNT level in the patient groups was lower than that of controls and inversely correlated with mean D-D in patients with thrombosis. This is an unreported finding which could reflect FNT incorporation into the fibrin network with resulting low plasma levels. On the other hand, low plasma FNT may adversely affect fibrin clearance by the reticulo-endothelial system allowing the persistence of elevated fibrin levels in plasma and/or on the vessel wall (38). Previous reports on fibrinolysis in the APS pointed towards a hypofibrinolytic state characterized by elevated PAI (12, 14, 15) a picture close to that of patients with idiopathic deep vein thrombosis (39). To overcome the inflammatory background of SLE or other autoimmune disorders which could influence the acute phase nature of PAI (40), the present study was carried out in subjects with idiopathic

aPLs. The higher mean basal tPA levels found in patients compared to controls, their within group correlation with D-D and the lack of differences in PAI levels apparently suggested a normo-functioning fibrinolytic system. However, gender analyzed data revealed that thrombotic females had blunted tPA release and higher mean level of PAI than thrombotic males and control females. Whether these abnormalities are due to the presence of aPL or to thrombosis per se remains unsettled, although epidemiological surveys outside the setting of APS have demonstrated that elevated levels of tPA and PAI in patients with a history of thrombosis may predict recurrences, implying that thrombosis may affect levels of tPA and PAI beyond the acute event (41). However, also non thrombotic females had higher mean PAI levels than thrombotic males and than control females. In this case the difference can only be accounted for by the presence of aPL.

A suggestive explanation exists to interpret our fibrinolysis data. In a normal population PAI increases with advancing age in females but not in males (42) the opposite occurs for tPA (43). The variations observed in the levels of PAI and tPA throughout our control and patient groups (of similar mean age) may simply indicate that aPL are prematurely emphasizing the age and sex dependency of these fibrinolytic variables. In conclusion, the results of the present study indirectly confirm the inhibitory action of aPL on the AT-HS system, highlight the association between acquired f-PS deficiency and increased thrombin generation even in asymptomatic carriers of aPLs (12, 15) and support a clustering of low f-PS with fibrinolysis defects. As to the mechanism of acquired f-PS deficiency, recent observations suggest that in the presence of aPL, C4b binding protein increases its affinity for PS, lowering plasma free form levels (44). Therefore f-PS deficiency may mediate the thrombotic tendency of the APS, but because of the acquired nature of the defect, its persistence should be serially ascertained (12). Further information regarding impaired fibrinolysis in individuals with aPL is provided, but a causal relationship with thrombosis could not be defined.

Effect of FXIIIa

To further our research on fibrinolysis we explored the role of factor XIII, a transglutaminase that promotes primarily fibrin-fibrin and fibrin-fibronectin cross-linking, followed by cross-linking with other haemostatic molecules in a hierarchical order, stabilizing the forming clot on the endothelial surface and contributing to cell adhesion (7). FXIIIa was particularly elevated in a proportion of our thrombotic PAPS patients, contributing to the higher level of this group compared to thrombotic and non-thrombotic controls. This would be in keeping with the concept that only thrombotic subjects had undergone activation of the coagulation system, clot stabilization, followed eventually by plasmin digestion. Indeed FXIIIa positively correlated to FNG, substrate as fibrin of FXIIIa, and PAI antigen, a surrogate of depressed fibrinolysis, in both PAPS and thrombotic controls. Given that fibrinogen and PAI were higher in PAPS, the overall picture is one of increased FXIII activity contributing to fibrin cross-linking in a state of heightened fibrin turnover in PAPS (45). As result of enhanced cross-linked, fibrin is less amenable to plasmin digestion, and conceivably a fibrin film remains adherent to and maintains the endothelial surface in persistent activation (46). Indeed, when re-assessed, FXIIIa well correlated to baseline measurements in a subset of our PAPS patients and thrombotic controls.

Activation of FXIII requires the detachment of its B subunit under the action of thrombin (7), the generation of which is heightened in primary APS (45). Interestingly the B subunit of factor XIII contains a number of Sushi domains (7), the same domains present on β 2GPI, target of aPL (47). The relations between FXIIIa and IgG anti- β 2GPI in our antiphospholipid positive groups may indicate a regulatory role for IgG anti β 2GPI in FXIII activation.

CONCLUSION

These data confirm that lupus anticoagulant assays are better related to a history of thrombosis than IgG aCL: DRVVT being able to identify patients with a history of arterial

thrombosis, whereas IgG aCL levels correlated to the number of venous but not arterial events. This further supports the hypothesis that IgG aCL and LA are antibodies with distinct specificities and functional properties (48) and they may differentially associate with the risk of developing arterial or venous occlusions. Some evidence is given that plasma levels of vWF and FNG are associated with the occurrence of thrombosis though the data presented are inconclusive as to whether raised plasma FNG and vWF represents a cause or an effect of endothelial injury or thrombosis, even considering that FNG and vWF were measured three months after the thrombotic episodes, well beyond the acute phase period.

Although these studies do not address the relationship between aPLs and atherosclerosis, a hot topic in the APS (49), the wealth of our data point towards accelerated fibrin turnover in patients with aPL, supported by the enhanced activity of factor XIII, and there is more than circumstantial evidence to recognise fibrin deposition on the vessel wall as an early phenomenon in atherogenesis (7).

The unsuspected sex dependency of fibrinolytic abnormalities, the hypothesis that aPLs might promote in younger subjects a fibrinolytic pattern typical of older populations, the differential effect of plasma FNG and vWF on arterial and venous occlusions and eventually atherosclerosis in PAPS are issues that only large prospective studies can evaluate.

CHAPTER V

LIPID PROFILE, LIPID PEROXIDATION AND ANTI-LIPOPROTEIN ANTIBODIES IN THROMBOTIC PRIMARY ANTIPHOSPHOLIPID SYNDROME

INTRODUCTION

Lipoprotein imbalance in atherosclerosis

Dyslipidaemia is a highly prominent atherogenic risk factor intimately associated with premature atherosclerosis. Excess plasma levels of circulating cholesterol under the form of pro-atherogenic ApoB containing lipoproteins with subnormal levels of anti-atherogenic ApoAI containing lipoproteins represents the major dyslipidaemic imbalance. ApoB is the predominant protein component of pro-atherogenic, cholesterol-rich LDL, TG-rich VLDL, VLDL remnants and intermediate-density lipoprotein (IDL), whereas ApoA-I is the major protein component of anti-atherogenic HDL. Elevated serum concentration of LDL-cholesterol (LDL-C) was considered the most common form of atherogenic dyslipidemia (1), but it is recognized that also a low serum concentration of HDL-cholesterol (HDL-C) is a definite atherogenic trait (2). Several epidemiological studies have found that low serum HDL-C concentrations (defined as <40 mg/dl in both sexes or as <40 mg/dl in men and <50 mg/dl in women) (3,4) constitute an independent risk factor for coronary heart disease in both non diabetic and diabetic subjects (2-4). Prospective studies have revealed that a decrease of 1 mg/dl in HDL-C increases the cardiovascular risk by 3% in women and by 2% in men (1). Conversely, subjects with elevated HDL-C levels show a decreased risk of CV events (2, 3-5). The prevalence of low HDL-C concentration may vary from 20% in a general population to up to 60% in patients with established cardiovascular disease (CVD) (6). Not only low HDL-C concentration associates with an increased incidence of CVD but also with a more

aggressive progression of angiographically defined coronary artery disease (CAD), a greater risk for carotid atherosclerosis and ischemic stroke mortality (2,3). The Myocardial Ischemia Reduction with Aggressive Cholesterol Lowering (MIRACL) trial showed that baseline HDL-C levels, rather than LDL-C levels predicted the re-occurrence of CVD in patients with acute coronary syndromes treated with atorvastatin (7).

Oxidant/antioxidant balance in atherosclerosis

Oxidative stress is defined as an imbalance between pro-oxidant and antioxidant factors in favor of pro-oxidants and is central to atherogenesis and its cardiovascular consequences (8). Analysis of plaque composition has revealed products of protein and lipid oxidation, such as oxidized and nitrated amino acids, lipid hydroperoxides, short-chain aldehydes, oxidized phospholipids, F2-isoprostanes, and oxysterols, suggesting the local occurrence of oxidative stress (9). The preferential retention of LDL in the arterial wall makes LDL a major pro-oxidative substrate within the arterial intima. Various oxidative systems including NAD(P)H oxidases, xanthine oxidase, myeloperoxidase, uncoupled nitric oxide synthase, lipoxygenases and the mitochondrial electron transport chain can generate reactive oxygen and nitrogen species that contribute to the in vivo oxidation of LDL (10).

This wide array of oxidative enzymes and the consequent oxidation of LDL is counteracted by the antioxidant activity of HDL; several enzymes such as paraoxonase (PON1) and phospholipase A2 that degrade lipid peroxides and a variety of lactones including homocysteine thiolactone are part of the anti-oxidant arsenal of HDL (11-13). However, if the antioxidant capacity of HDL is overridden, LDL will oxidise and generate different classes of toxic compounds, amongst which F2-isoprostanes and a variety of peroxides, hydroperoxides and aldehydes.

F2-isoprostanes are cyclic prostaglandin-like compounds formed through peroxidation of arachidonic acid present in lipoproteins and cell membranes in the absence of cyclo-

oxygenase: they are currently the most robust and integrative markers of oxidative stress in vivo (14), have strong vasopressor activity (15), may facilitate platelet activation (16), and are strong and independent risk factors for CHD (17, 18).

Further by products of LDL oxidation include 1-palmitoyl-2-sn-glycero-3-phosphorylcholine, 1-palmitoyl-2-glutaroyl-sn-glycero-3-phosphorylcholine, cholesteryl-linoleate-hydroperoxide, 7-hydroperoxycholesterol, hydroxyoctadecadienoic acid and 4-hydroxynonenal (8, 19).

Oxidised LDL and its ligands

On its own, oxLDL exerts pro-inflammatory activities by inducing the expression of adhesion molecules on endothelial cells, by attracting circulating monocytes on the same cells, and by promoting the release of chemokines and inflammatory cytokines from macrophages (8). Moreover oxLDL is taken up through macrophage scavenger receptors and lead to monocyte transformation into cholesterol-loaded macrophages then into foam cells, characteristic residents of atherosclerotic plaques (8).

The oxidative modification of LDL makes this lipoprotein immunogenic so to elicit a specific autoantibody response that cross-reacted with anticardiolipin in systemic lupus erythematosus (20): in fact some antiphospholipid antibodies can be directed against epitopes of oxidised phospholipids (21). The studies that have addressed the role of IgG anti oxLDL in vascular disease have yielded discordant results (22).

In an attempt to quench LDL oxidation, β_2 GPI binds oxLDL but not native LDL in vitro. Two chloroform-extractable lipids (oxLig-1 and oxLig-2) were identified as the LDL-derived ligands for the specific interaction between oxLDL and β_2 GPI that is initially only electrostatic but later progresses to a covalent bond via Schiff base formation (23). The oxLDL/ β_2 GPI complex is also immunogenic and elicits a specific antibody response. When IgG anti- β_2 GPI antibodies are co-incubated with oxLDL and β_2 GPI, the

monocyte/macrophage uptake and intracellular accumulation of oxLDL accelerates, likely via FcγRI receptors, leading also to up-regulation of the scavenger receptor CD36 and further surface expression of FcγRI receptors (23). Experiments evaluating the intracellular trafficking of β₂GPI within macrophages showed that un-complexed β₂GPI was poorly incorporated in late endosomes and stagnated there, whereas complexed β₂GPI (to phosphatidylserine liposomes or oxLDL) was quickly transported to lysosomes; the addition of antibodies to β₂GPI further accelerated this process (24).

EXPERIMENTAL DATA

1) THE LIPID PROFILE, ANTI LIPOPROTEIN ANTIBODIES AND C-REACTIVE PROTEIN.

The finding that oxLDL and the antibody response it stimulated were of low specificity (22) preceded the discovery that β₂GPI bound to oxLDL in a stable atherogenic oxLDL-β₂GPI auto-antigen (23). Although oxLDL-β₂GPI concentration is elevated in patients with SLE with and without APS neither occur in association with SLE disease activity nor with any major clinical manifestation of APS (24). At variance antibodies against oxLDL-β₂GPI occur in SLE and/or APS in relation to the intima media thickness of carotid arteries (25). More recently antibodies against HDL hampering the anti-atherogenic and anti-inflammatory properties of HDL have been detected in SLE (26).

This study investigated an extended lipid profile, the presence of antibodies against HDL and ApoA and their relationship with high sensitivity C-reactive protein (hs-CRP) in thrombotic patients with PAPS compared to patients with inherited thrombophilia (IT) and healthy controls. Hence the study was a cross-sectional case double-control: consecutive patients fulfilling criteria for PAPS (27) and patients with IT were invited to participate. Exclusion criteria were acute or chronic hepatic, renal, lung disease, diabetes, a recent history of acute infection (within six weeks), a positive urinary dipstick for nitrates on the day of

sampling, steroid, statin or fibrate treatment. Of 40 PAPS patients, one was excluded because had gradually developed systemic lupus erythematosus, one had developed kidney cancer, three were pregnant and one had died of a recurrent event. Of 38 IT patients one had had recent surgery and one was pregnant. The study therefore included 34 thrombotic PAPS patients (arterial occlusions n=10, venous occlusions n=19, arterial + venous occlusions n=5) 36 thrombotic IT patients (arterial occlusions n=5, venous occlusions n=28, arterial + venous occlusions n=3; factor V Leiden n=16, prothrombin 20210 n=10, protein C deficiency n=4, free protein S deficiency n=6) and 28 control subjects. In the PAPS group, 3 patients were on carbamazepine for post-ischemic epilepsy, two on beta-blockers and 3 were menopausal. In the IT group, 2 patients were on beta-blockers and 4 were menopausal. The control group was made up of 19 blood donors and 9 healthy hospital personnel: of these 3 women were menopausal. Table 1 shows the demographics of the three groups.

Table 1. Demographics of study groups.

	CTR	IT	PAPS
	(n=28)	(n=36)	(n=34)
F/M	16/12	18/18	24/10
Age	45±13	50±14	44±11
IgG aCL (GPL)	6.8±3.5	6.4±2.7	131±140
IgG β_2 GPI (IU)	2.3±0.8	4.2±0.8	118±67
Lupus anticoagulant	0	0	30

PAPS: primary antiphospholipid syndrome; IT: inherited thrombophilia;
CTR: control; aCL: anticardiolipin; β_2 GPI: beta-2-glycoprotein-I

RESULTS

Comparison of variables across groups

With regards to the lipid profile the PAPS group showed lower average HDL, LDL, total cholesterol and ApoAI concentrations other groups. With regards to antibodies against lipoprotein components the PAPS group showed higher IgG anti-HDL and IgG anti-ApoAI

titres than other groups. OxLDL- β_2 GPI and CRP were almost exclusively elevated in PAPS (Table 2).

Table 2. Lipid profile, anti-HDL, anti-ApoAI, oxLDL- β_2 GPI and CRP across groups

	CTR	IT	PAPS	p-value
	(n=28)	(n=36)	(n=34)	
HDL (mg/dl)	49 \pm 15	31 \pm 7.6	31 \pm 9.4	<0.0001
LDL (mg/dl)	118 \pm 28	93 \pm 26	82 \pm 23	<0.0001
CHO (mg/dl)	205 \pm 26	187 \pm 39	169 \pm 33	0.0002
TG (mg/dl)	83 \pm 29	94 \pm 42	108 \pm 52	0.01
ApoAI (mg/dl)	1.5 \pm 0.23	1.3 \pm 0.23	1.2 \pm 0.25	0.002
ApoB (mg/dl)	0.9 \pm 0.24	0.8 \pm 0.25	0.8 \pm 0.21	NS
IgG anti-HDL (% PC)	100 \pm 105	93 \pm 81	192 \pm 208	0.006
IgG anti-ApoAI (% PC)	31 \pm 16	32 \pm 24	91 \pm 76	<0.0001
Ox LDL- β_2 GPI (U/ml)	2.8 \pm 4.0	2.9 \pm 3.0	6.4 \pm 7.0	0.001
hs-CRP (mg/dl)	0.22 \pm 0.33	0.26 \pm 0.2	0.66 \pm 0.8	0.003

PAPS: primary antiphospholipid syndrome; IT: inherited thrombophilia; CTR: control; HDL: high density lipoprotein; LDL: low density lipoprotein; CHO: cholesterol; ApoA: apolipoprotein AI; ApoB: apolipoprotein B; PC: positive control; oxLDL- β_2 GPI: oxidized LDL/beta-2-glycoprotein-1; hs-CRP: high sensitivity C-reactive protein.

Relationship amongst variables in the PAPS group

By univariate analysis only IgG anti-HDL correlated to HDL (Figure 1). Several variables correlated to oxLDL- β_2 GPI, notably LDL, ApoB and IgG aCL (Table 3). A multiple regression model with oxLDL- β_2 GPI as the dependent variable and the lipid fractions as independent did not identify independent predictors as most of the independent variables were interrelated. A similar model with oxLDL- β_2 GPI as dependent variable and IgG aCL, IgG β_2 GPI, IgG anti-HDL and IgG anti-ApoAI as independent variables identified IgG aCL and

IgG anti-HDL as predictors of oxLDL- β_2 GPI (Table 4). By univariate analysis HDL and ApoAI negatively correlated to hs-CRP ($r=-0.42$, $p=0.01$ and $r=-0.49$, $p=0.002$) respectively.

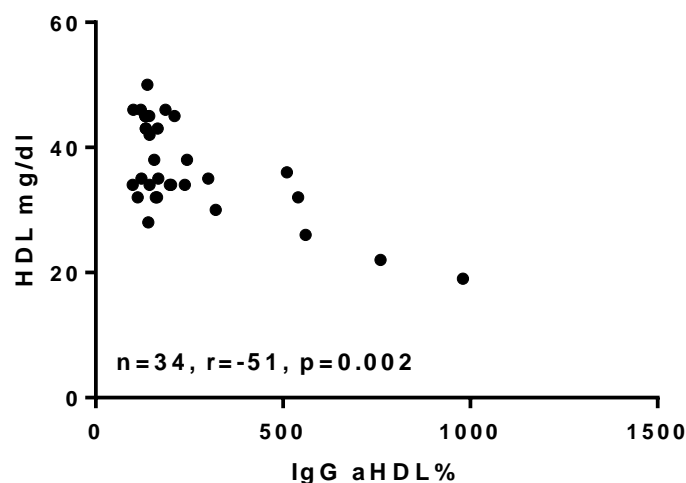


Figure 1. Spearman rank correlation of high density lipoprotein (HDL) with antibody against HDL in patients with thrombotic antiphospholipid syndrome.

Table 3. Determinants of oxLDL- β_2 GPI in PAPS

Variable	R	P-value
IgG aCL	0.41	0.006
IgG β_2 GPI	0.34	0.02
IgG anti-HDL	0.38	0.01
ApoB	0.45	0.006
CHO	0.35	0.04
HDL	-0.34	0.04
LDL	0.43	0.008
BMI	0.38	0.01

PAPS: primary antiphospholipid syndrome; oxLDL- β_2 GPI: oxidized LDL/beta-2-glycoprotein-1; aCL: anticardiolipin; β_2 GPI: beta-2-glycoprotein-I; CHO: cholesterol; ApoB: apolipoprotein B; HDL: high density lipoprotein; LDL: low density lipoprotein; BMI: body mass index.

Table 4. Independent predictors of oxLDL- β_2 GPI in PAPS

	B	t	p-value
IgG aCL	0.575	3.653	0.001
IgG anti-HDL	0.355	2.751	0.009

oxLDL- β_2 GPI: oxidized LDL/beta-2-glycoprotein-1; PAPS: primary antiphospholipid syndrome; aCL: anticardiolipin; anti-HDL: anti-high density lipoprotein antibodies.

Relationship amongst variables in the IT group

By univariate analysis oxLDL- β_2 GPI correlated to LDL ($r=0.43$, $p=0.009$) and anti-HDL antibodies ($r=0.49$, $p=0.002$) in the IT group.

Relationship amongst variables in CTR

By univariate analysis oxLDL- β_2 GPI correlated negatively to HDL ($r=-0.71$, $p<0.0001$) and to ApoAI ($r=-0.49$, $p=0.01$) and positively to hs-CRP ($r=0.59$, $p=0.0001$). hs-CRP negatively correlated to HDL ($r=-0.59$, $p=0.001$).

2) OXIDATIVE STRESS AS A RESULT OF LDL OXIDATION

The presence of oxLDL, oxLDL- β_2 GPI and their specific antibodies in the circulation of patients with autoimmune disorders was the drive to assess the occurrence of lipid peroxidation in PAPS when the methodology became available. Oxidative stress had been detected in several autoimmune vascular disorders including systemic lupus erythematosus (28) but not in patients with PAPS. In a pilot study plasma 8-epi-prostaglandin F₂ α was significantly higher in 10 patients with PAPS (6M, 4F, mean age 35 ± 15 years) than 10 age (± 3 years) and sex matched healthy controls (234 ± 56 pg/ml vs 72 ± 14 pg/ml, $p=0.0002$) and strongly related to the plasma IgG aCL titre ($r=0.89$, $p=0.0004$)(29). This led to the second study partially reported here in which F₂-isoprostanes were measured alongside two other vasoactive molecules, TXB₂ and endothelin-1 (ET-1) in 14 persistent carriers of aP)

(8F, 6M, mean age 39 ± 11 y, mean IgG aCL 187 ± 42 GPL) and on 12 (6F, 6M, mean age 36 ± 11 y) healthy hospital personnel who served as a control group. A history of thrombosis was present in 10 aPL patients who fulfilled thus criteria for PAPS (27) whereas four participants were persistent carriers of aPL who never suffered thrombosis. None of the aPL participants had systemic lupus erythematosus or any other underlying disease. Neither patients nor controls smoked. Of the 10 APS participants, nine were on warfarin at the time of study and one was on aspirin (75 mg/day).

RESULTS

Baseline comparison between antiphospholipid patients and controls

APL participants showed enhanced urinary excretion of F₂-isoprostanes and of TxB₂ as well as higher plasma levels of ET-1 than control participants (Table 5). In the aPL group, IgG aCL titre positively related to urinary TxB₂ ($r=0.72$, $p=0.01$).

Table 5. F₂-isoprostane and vasoactive molecules in PAPS and controls.

	Control (n=12)	PAPS (n=14)	p-value
Age (mean±SD)	36±11	39±11	
Sex F/M	6/6	6/8	
Cr clearance (ml/s)	1.95±0.19	1.85±0.27	
ET-1 (pg/ml=	0.77±0.08	2.09±0.2	0.0002
TxB ₂ (pg/mmol Cr)	11.6±2.4	42.2±6.8	0.0004
F ₂ -isoprostane (ng/mmol Cr)	0.139±0.03	0.218±0.02	0.02

aPL: antiphospholipid antibody; Cr: creatinine; ET-1: endothelin-1; TxB₂: 11-dehydro-thromboxane B₂.

DISCUSSION

Thrombotic PAPS were characterised by low plasma concentration of HDL and of its major apolipoprotein: whilst this has relevance to venous occlusions (30) and its re-occurrence in non autoimmune populations (31) as in our IT patients, it did not discriminate arterial or venous thrombosis in our PAPS group. Of the previous surveys two showed lower mean HDL in PAPS patients (32, 33) while a third did not (34, 35). On the other hand average total cholesterol was reported elevated in two studies (32, 34) and low in prevalence (36) and average (33) in other two studies as in ours.

With regards to LDL three studies found elevated mean levels in PAPS (32-34) whereas we found lower levels than controls. In the presence of an oxidant environment LDL oxidises and releases lipid radicals with powerful biological properties: β_2 GPI binds and neutralises oxLDL in a stable oxLDL/ β_2 GPI complex (23): supportive of these in vitro data LDL and HDL related positively and negatively to oxLDL- β_2 GPI, in keeping with the notion that β_2 GPI may buffer excessive LDL oxidation determined by the loss of the antioxidant capacity of HDL residing mostly in its paraoxonase activity (26).

Indeed antibodies against HDL and ApoAI are associated with decreased paraoxonase activity in vitro and in vivo (26): here they were present only in the PAPS group where IgG anti-HDL negatively correlated to HDL and independently predicted oxLDL- β_2 GPI levels. This confirms that IgG anti-HDL may hamper the antioxidant effect of HDL favouring LDL oxidation therefore increased oxLDL- β_2 GPI formation and enhanced oxidative stress measured as F2-isoprostanes in our PAPS patients.

With regards to the reduced average HDL concentration of our PAPS group one cannot exclude enhanced clearance of a putative IgG anti-HDL-HDL complex. Moreover HDL showed a negative, albeit modest correlation with hs-CRP, indicating that loss of the anti-inflammatory effect of HDL may contribute to the recently described low-grade inflammation of PAPS patients (37). Interestingly the average triglyceride concentration was higher in our

PAPS group: this is similar to the picture of SLE where an inhibitory activity on lipoprotein lipase accounts for decreased hydrolysis of circulating triglycerides (38). In conclusion elevated triglycerides and low HDL in association with antibodies against HDL seems a common finding in thrombotic PAPS and may predispose to enhanced oxidative stress. Studies assessing the atherothrombotic risk of PAPS populations should take into account this interface between traditional and non traditional cardiovascular risk factors.

CHAPTER VI

NITRIC OXIDE AND NITRATIVE STRESS IN THROMBOTIC PRIMARY ANTIPHOSPHOLIPID SYNDROME

INTRODUCTION

Nitric oxide and the vasculature

Nitric oxide (NO) is an uncharged free radical composed of seven electrons from nitrogen and eight electrons from oxygen that acts as a signalling molecule within a source cell or diffuses from the source cell to affect adjacent cells (1-3). Within the vasculature, NO is produced by endothelial cells, smooth muscle cells, monocytes, neutrophils, eosinophils and platelets (4). Endothelial cells generate NO from two related nitric oxide synthases (NOS), endothelial/constitutive eNOS and inducible NOS (iNOS): both convert L-arginine to NO• and citrulline at different concentrations according to substrate availability (5). Although the physiological vascular roles of NO include modulation of vascular tone, inhibition of platelet function and angiogenesis, NO also modulates monocyte/macrophage that in turn may contribute to pathological vascular tone (4,6).

Under physiological conditions, the shear force of flowing blood stimulates endothelial receptors (7-8) that trigger calcium influx hence the eNOS activation. Under pathological conditions, bacterial endotoxins and inflammatory cytokines, such as TNF-alpha and interleukins, activate iNOS which produces calcium independent NO at a 1,000-fold rate greater than eNOS not only from endothelial and smooth muscle cells but also from infiltrating monocytes, lymphocytes and fibroblasts (9).

Nitric oxide and smooth muscle cells

NO controls smooth muscle cells contractility by modulating the activity of heme containing soluble guanylyl cyclase (sGC) that de-phosphorylates guanosine triphosphate

(GTP) to cyclic guanosine 3',5'-cyclic monophosphate (cGMP). This pathway inhibits calcium entry into smooth muscle cells and allows phosphorylation of myosin light chains and of sarcoplasmic proteins, leading to calcium sequestration in the sarcoplasmic reticulum (10-11) and ultimately leads to smooth muscle cell relaxation (12). Similarly in platelets, NO affects cGMP-dependent protein kinase activation that results in decreased intracellular calcium, an important second messenger in platelets function (13-16). In fact a decrease of intracellular calcium antagonises several calcium dependent enzymes involved in platelet aggregation, shape change and release of storage granules (16). Partly through down regulation of protein kinase C, cGMP may desensitize the thromboxane A2 (TXA2) receptor, preventing TXA2 mediated platelet aggregation and vasoconstriction (16).

Nitric oxide bio-reactivity

After eNOS generation, NO binds to the iron (III) hemes of cytochrome c oxidase in mitochondria, regulates certain transcription factors such as hypoxia-inducible factor-1 (2). Alternatively it diffuses into the vascular lumen where it is scavenged by the ferrous iron (Fe^{2+}) in the heme moiety of oxy-haemoglobin to form met-haemoglobin and nitrate (NO_3^-) (2, 3, 17).

NO can exist in three closely related redox forms: the free radical $\text{NO}\bullet$; NO^+ or nitrosonium and NO^- or nitroxyl anion, resulting from a one-electron reduction of $\text{NO}\bullet$. NO can be reduced to nitrous oxide (N_2O) or oxidized to nitrite (NO_2^-) (18). NO_2^- reacts rapidly with oxygen, yielding nitrogen dioxide radical ($\bullet\text{NO}_2$), which exists in equilibrium with the potent nitrosating agents dinitrogen trioxide (N_2O_3) and dinitrogen tetroxide (N_2O_4); $\bullet\text{NO}_2$ can either react with unsaturated lipids directly or participate in nitrosation reactions. S-nitrosation may generate S-nitrosothiols that due to their stability represent a storage pool of bio-available NO (19). In plasma, S-nitrosoalbumin is an important NO adduct that may protect NO from inactivation in the oxidative extracellular milieu (20).

At low concentrations, intracellular NO may function as an antioxidant through termination reactions with lipid radicals ($L\cdot$, $LO\cdot$, $LOO\cdot$), resulting in the formation of less reactive secondary nitrogen-containing products (LONO, LOONO) (1). LOONO can either decompose to caged radicals ($LO\cdot$ $\cdot NO_2$) with rearrangement of $LO\cdot$ to an epoxide ($L(O)NO_2$), dissociate and react with additional NO, or hydrolyze to LOOH and nitrite NO_2^- (18). By these reactions, NO suppresses the generation of lipid-derived chemo-attractants for monocytes and can thereby be considered an anti-inflammatory and potentially anti-atherosclerotic molecule (21, 22).

Nitric oxide and nitrate stress

The oxidative inactivation of NO by superoxide anion ($O_2^{\cdot-}$) is the prevalent mechanisms leading to decreased bioavailability of NO, impaired vasomotor tone and enhanced platelet function. Reactive oxygen species (ROS) and $O_2^{\cdot-}$ from monocytes, neutrophils, endothelial cells and platelets react with NO at physiological pH to generate peroxynitrite anion ($ONOO^-$) that occurs at a faster rate (23,24) than that of the SOD reaction or of NO with heme compounds (25). Thus, NO can be considered a scavenger of $O_2^{\cdot-}$ even to the extent of acting as an antioxidant; in fact, NO is the only biological molecule that can kinetically out-compete SOD for $O_2^{\cdot-}$ (26-28). Exogenous cigarette smoke also yields high levels of NO that significantly contributes to peroxynitrite formation (29).

In vivo peroxynitrite may nitrate protein tyrosinyl residues to yield 3-nitrotyrosine though tyrosine does not react directly with peroxynitrite but a hydrogen atom is first abstracted from the phenol ring to form a tyrosyl radical that then combines with $\cdot NO_2$ to produce 3-nitrotyrosine (30). Alternatively peroxynitrite may reaction with carbon dioxide radical ($CO_2\cdot$) to form highly reactive nitrosoperoxy carbonate anion ($ONO_2CO_2^-$) (31) that may also nitrosate substrates. Peroxynitrite itself is not a direct nitrosating agent, but is involved in the nitrosation of thiols to yield RSNO or organic nitrates (30, 32, 33). In addition, CO_2 may

enhances tyrosine nitration by peroxynitrite in vitro (34). Immunodetectable 3-nitrotyrosine has been demonstrated in fatty streaks of coronary arteries of young autopsy subjects and in foam cells, vascular endothelium, and the neointima of advanced atherosclerotic lesions in older patients and as such is a specific marker of nitrative stress (35).

As a lipid oxidant, peroxynitrite produces lipid peroxyl radicals and mediates peroxidation of diverse classes of lipids including phospholipids and LDL lipids, forming conjugated dienes, malondialdehyde, lipid peroxides, lipid hydroxides and F2-isoprostanes (36, 37-38). The peroxynitrite induced oxidation of free thiol residues in protein, lipids, deoxyribose, guanine bases, methionine, and phenols leads to protein modification and inhibition of enzymatic activities (39-46). By the same token, peroxynitrite may have profound effects on cell signalling modifying target proteins involved in signal transduction (47).

EXPERIMENTAL DATA

1) NITRIC OXIDE AND NITRATIVE STRESS IN PRIMARY THROMBOTIC ANTIPHOSPHOLIPID SYNDROME

The role of NO in PAPS had never been fully appreciated: the few available studies were too limited in numbers to provide a full understanding of its significance (48,49). However a decreased NO• was detected in animal models of APS (50,51) supporting the possibility that NO• might be involved in the vascular pathogenesis of PAPS. To expand on this topic the behaviour of NO• metabolites nitrite (NO_2^-) and nitrate (NO_3^-), total antioxidant capacity (TAC) (expressed as ONOO^- quenching), and nitrotyrosine (NT) was compared in patients with thrombotic PAPS, in asymptomatic but persistent carriers of aPL, in patients with IT with vascular occlusions, in patients with SLE, and in healthy subjects. Possible relationships between NO_2^- , NO_3^- , hs-CRP, and several aPL were also investigated.

MATERIALS AND METHODS

Patients.

Our study was devised as a cross-sectional case-quadruple control: PAPS patients with vascular occlusions represented cases; IT patients with vascular occlusions represented thrombotic controls, aPL without vascular occlusions represented non-thrombotic aPL-positive controls; patients with SLE represented inflammatory controls and healthy subjects represented normal controls. All participants were age- and sex-matched (where possible), except for SLE patients, who were all female. Consecutive patients with thrombotic PAPS, with IT and persistent aPL attending the Coagulation Unit of the Cardarelli Hospital (Naples, Italy) were invited to participate between January 2008 and July 2008. Our study was carried out according to the revised Declaration of Helsinki, with approval of the Ethics Board of the hospital and written consent of all participants. Exclusion criteria were acute or chronic hepatic, renal, and lung disease; diabetes; acute infection (within 6 weeks); post-thrombotic syndrome with or without venous ulcerations; positive urinary dipstick for nitrites on the day of sampling and treatment with statins or fibrates. PAPS and IT patients are seen on average every 3 to 4 weeks for oral anticoagulation monitoring and are instructed to self-report any illness during the intervening periods; their lipid profiles and kidney and liver function tests are checked annually. APL subjects were diagnosed as such either because of the presence of prolonged clotting tests in routine assays, subsequently confirmed as lupus anticoagulants (LAC), or because of thrombocytopenia or other symptoms that prompted a search for aPL. Of the PAPS attendees (n=50), 2 were excluded because they had gradually developed ankylosing spondylitis and SLE, one had developed kidney cancer, 2 were pregnant, one had suffered a recent recurrent event, one had post-thrombotic syndrome, and 2 were evasive regarding their smoking and contraceptive status. Of the IT (n=46) attendees, 2 were excluded for post-thrombotic syndrome and venous ulcerations in lower limbs. Of the aPL attendees (n=27) one was excluded for development of non-insulin-dependent diabetes; one for the

development of SLE, haemolytic anemia, nephrotic syndrome, and pulmonary embolism after ovarian hyperstimulation; one for the development of chronic lymphoid leukemia; one for spontaneous onset of ischemic stroke; and 2 had moved to a different town. Of the remaining aPL subjects 4 had moderate thrombocytopenia (platelets $< 100 \times 10^9/l$) not requiring treatment.

Consecutive patients with SLE fulfilling the American Rheumatism Association (ACR) criteria (52) were enrolled among those attending the Autoimmune Outpatient Clinic of the Curry Cabral Hospital, Lisbon (Portugal) between January 2008 and August 2008. Exclusion criteria included acute or chronic renal impairment that would significantly alter NO metabolites, liver cirrhosis, diabetes, acute infection (within 6 weeks), post-thrombotic syndrome with or without venous ulcerations, positive urinary culture following positive dipstick for nitrite (urinary excretion in SLE may be increased in the absence of infection), and treatment with statins or fibrates. Of 52 patients with SLE, 16 were excluded on the basis of the above criteria. Of the remaining 36, one was found weeks later to have tuberculosis and 2 were pregnant; their samples were discarded. Therefore 33 SLE patients participated in the study: of these, 9% had visceral involvement without renal disease, 12% cardiac and lung involvement, 9% central nervous system involvement, 66% arthritis, 3% myositis, 9% alopecia, 3% haemolytic anemia, 21% thrombocytopenia, 9% neutropenia, 81% presence of anti-DNA antibodies, and 90% presence of antinuclear antibodies. The average SLE Disease Activity Index (SLEDAI) score was 4.85 ± 3.96 (median 3.5, range 0–16). Their medication intake was: prednisolone in 54%, (< 6 mg/day in 27%, 6–10 mg/day in 18%, > 10 mg/day in 9%) azathioprine in 24% (100 mg/day in 18%, 150 mg/day in 6%), hydroxychloroquine 200 mg/day in 60%, aspirin in 12%, warfarin in 9%. Twenty-nine healthy hospital staff served as normal controls: 15 from Cardarelli Hospital in Naples and 14 from the Curry Cabral Hospital in Lisbon. To minimize dietary influences on nitric oxide metabolite concentrations all

participants were asked to refrain from foodstuffs containing high concentrations of nitrate/nitrite (such as lettuce, spinach, beetroot, radish, salamis, and pickled items) for 3 days before blood sampling. The study was therefore carried out on 46 thrombotic PAPS patients, 21 aPL subjects, 38 IT patients, 33 SLE patients, and 29 control subjects. Their demographics are shown in Table 1.

Table 1. Demographics of participants across groups

	CTR	IT	aPL	PAPS	SLE
No	29	38	21	46	33
Age (mean±SD)	41±7	41±14	40±9	43±12	40±12
Sex M/F	15/14	13/25	6/15	18/28	0/33
Disease duration, median (range)		12 (1, 22)	10 (1, 16)	10 (1, 18)	8 (1, 13)
LA	0	0	44	18	6
IgG aCL median (range)	8 (2, 18)	8.4 (4, 20)	24 (2, 309)	100 (5, 500)	62 (12, 112)
IgG aβ ₂ GPI U/ml median (range)	4 (2.5, 8.2)	5 (2, 12)	4 (2, 90)	146 (2.6, 184)	12 (3.6, 102)
IgG anti-HDL % median (range)	61 (19, 161)	70 (22, 292)	112 (28, 240)	70 (25, 436)	98 (25.4, 328)
IgG anti-ApoAI % median (range)	0.18 (0.06, 0.57)	0.14 (0.02, 1.2)	1.2 (0.17, 7.4)	0.69 (0.17, 6.3)	1.2 (0.17, 6)
MTHFR +/+	5	5	6	13	4
PT20210	2	4	1	2	0
FVL	1	12	0	2	1
f-PS deficiency	0	8	4	32	NA
FVL + PT20210	0	7	0	0	0
FVL + PS deficiency	0	2	0	0	0
Thrombosis					
Arterial	0	11	0	14	4
Venous	0	27	0	26	1
Arterial+venous	0	0	0	6	0
No 1	0	38	0	19	4
No 2	0	0	0	16	0
No 3	0	0	0	3	0
No 4	0	0	0	2	0
Smokers					
<6 per day	4	4	3	6	2
7-15 per day	6	8	2	5	5
>15 per day	2	1	0	4	1

Abbreviations: PAPS: primary antiphospholipid syndrome; aPL: persistent carriers of antiphospholipid antibody with no underlying disorder and no thrombosis; IT: inherited thrombophilia; SLE: systemic lupus erythematosus; CTR: normal controls; LA: lupus anticoagulant; aCL: anticardiolipin; β₂GPI: beta-2-glycoprotein-I; anti-HDL: anti-high density lipoprotein; anti-ApoA-I: anti apolipoprotein A-I; MTHFR: homozygous methylen-tetrahydrofolate reductase C667T thermolabile mutation; PT: prothrombin; FVL: factor V Leiden; f-PS: free protein S.

RESULTS

Comparison of variables in PAPS, aPL, IT, SLE, and healthy controls.

Average plasma NO_2^- was lower in the PAPS, aPL, and IT groups (Figure 1A), whereas NO_3^- was higher in SLE (Figure 1B) and NT was higher in SLE and PAPS (Figure 1C).

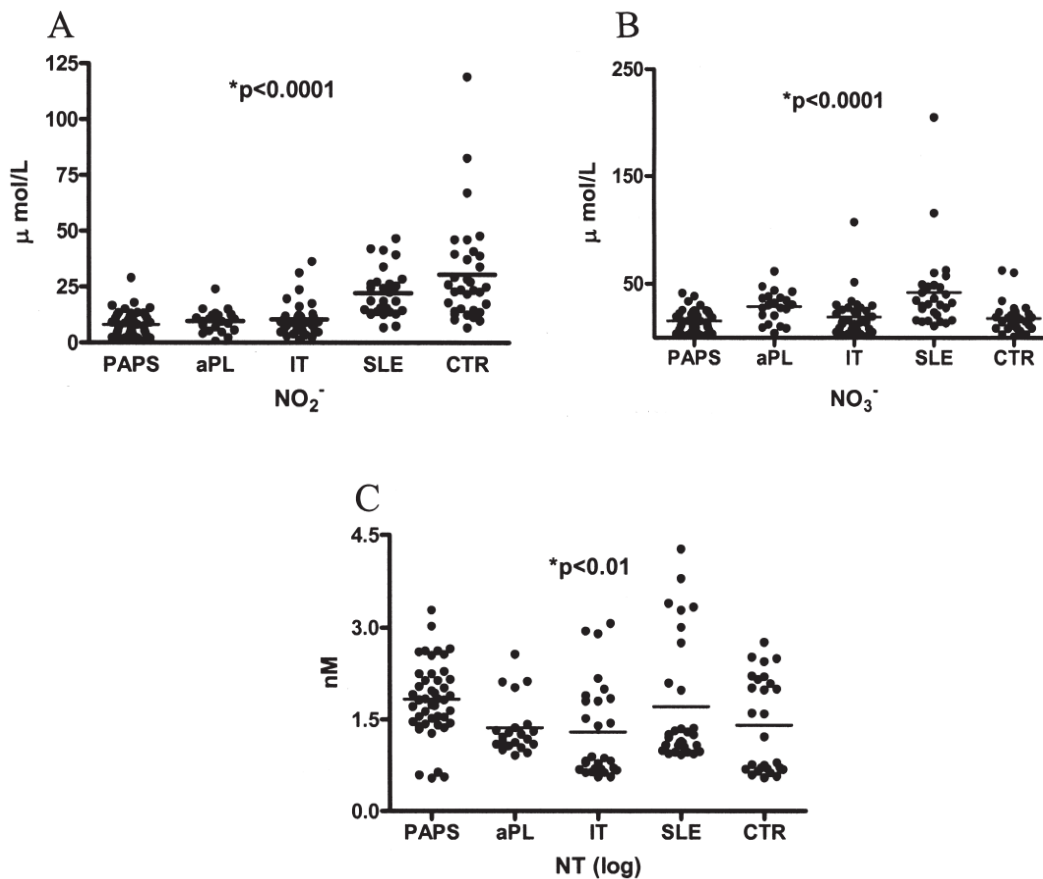


Figure 1. Comparison (ANOVA) of nitrite (NO_2^-), nitrate (NO_3^-), and nitrotyrosine (NT) across study groups: (A) average concentration of NO_2^- (Bonferroni's multiple comparison test: PAPS vs SLE, $p < 0.001$; PAPS vs CTR, $p < 0.001$; PCaPL vs SLE, $p < 0.01$; aPL vs CTR, $p < 0.001$; IT vs SLE, $p < 0.01$; IT vs CTR, $p < 0.001$); (B) average concentration of NO_3^- (Bonferroni's multiple comparison test: PAPS vs SLE, $p < 0.001$; IT vs SLE, $p < 0.001$; SLE vs CTR, $p < 0.001$); (C) average concentration of NT (Bonferroni's multiple comparison test: PAPS vs IT, $p < 0.05$). PAPS: primary antiphospholipid syndrome; PCaPL: persistent carriers of antiphospholipid antibodies without thrombosis or miscarriages; IT: inherited thrombophilia; SLE: systemic lupus erythematosus; CTR: controls.

Mean plasma TAC was lowest in SLE (Figure 2A), where CRP was highest (Figure 2A and 2B). Average TAC was higher in males than in females in all non-SLE groups: in PAPS 11280 ± 3041 versus 9749 ± 2967 $\mu\text{mol/l}$ ($p=0.02$); in IT 6392 ± 1399 versus 5325 ± 1720 $\mu\text{mol/l}$ ($p=0.04$); and in healthy controls 7967 ± 991 vs 6194 ± 1265 $\mu\text{mol/l}$ ($p=0.001$). Age, sex, and smoking correlated to NO_2^- , NO_3^- and TAC but had no confounding effect on resulting significance findings by ANCOVA. Age and IgG aCL related to NT and their confounding effect by ANCOVA reduced the comparative significance ($p < 0.02$). Age, sex, and smoking correlated to NO_2^- , NO_3^- and TAC but had no confounding effect on resulting significance findings by ANCOVA. Age and IgG aCL related to NT and their confounding effect by ANCOVA reduced the comparative significance ($p < 0.02$).

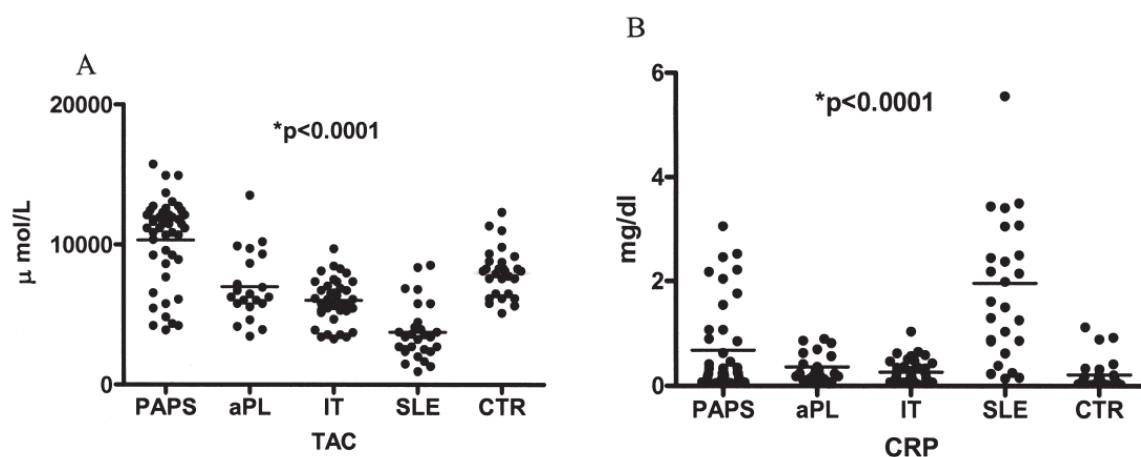


Figure 2. Comparison (ANOVA) of total antioxidant capacity (TAC) and high sensitivity C-reactive protein (CRP) across study groups: (A) average concentration of TAC (Bonferroni's multiple comparison test: PAPS vs IT, $p < 0.001$; PAPS vs SLE, $p < 0.001$; IT vs SLE, $p < 0.05$; IT vs CTR, $p < 0.05$; SLE vs CTR, $p < 0.001$); (B) average concentration of CRP (Bonferroni's multiple comparison: SLE vs PAPS, SLE vs aPL, SLE vs IT, SLE vs CTR, all $p < 0.001$). PAPS: primary antiphospholipid syndrome; PCaPL: persistent carriers of antiphospholipid antibody positive without thrombosis or miscarriages; IT: inherited thrombophilia; SLE: systemic lupus erythematosus; CTR: controls.

Relationship among variables in PAPS

The effect of antibodies and that of other clinical and laboratory variables on plasma concentrations of NO_2^- , NO_3^- , and NT was tested by separate multiple regression models. In the model with NO_2^- as the dependent variable and IgG aCL, IgG anti- β_2 GPI, IgG anti-HDL, and IgG anti-ApoAI antibodies as independent variables, IgG aCL resulted in the only negative predictor of NO_2^- ($p = 0.03$; Table 2). Average NO_2^- was lower in patients with a history of arterial thrombosis versus those with venous thrombosis (11.41 ± 7.6 vs 18.43 ± 11.06 $\mu\text{mol/l}$; $p=0.03$) although in a separate model with NO_2^- as the dependent variable and age at first thrombotic event and thrombosis number and type as the independent variables, thrombosis number negatively predicted NO_2^- ($p=0.001$; Table 2). In the model with NO_3^- as the dependent variable and IgG aCL, IgG anti- β_2 GPI, IgG anti-HDL, and IgG anti-ApoAI antibodies as independent variables, IgG aCL was the only negative predictor of NO_3^- ($p = 0.03$) (Table 2). In a different model, with NO_3^- as the dependent variable and age, sex, thrombosis type, smoking, and TAC as the independent variables, arterial thrombosis and smoking independently predicted NO_3^- ($p=0.05$ and $p=0.005$, respectively). In a further model with NT as the dependent variable and the antibodies as the independent variables none of the latter bore any relationship with NT; but in a similar model NT as the dependent variable and with NO_3^- , NO_2^- , TAC, smoking, and CRP as independent variables, CRP was the only independent predictor of NT ($p=0.004$) (Table 2).

Table 2. Predictors of nitric oxide metabolites and nitrotyrosine in PAPS.

Variables in regression model				
Independent	Dependent	Predictors	t	p-value
IgG aCL, IgG a β_2 GPI, IgG anti-HDL, IgG anti-ApoAI	NO $_2^-$	IgG aCL	-1.87	0.03
Age at first thrombosis, thrombosis number & type	NO $_2^-$	Thrombosis number	-3.24	0.001
IgG aCL, IgG a β_2 GPI, IgG anti-HDL, IgG anti-ApoAI	NO $_3^-$	IgG aCL	-1.93	0.03
Age, sex, thrombosis type, smoking, TAC	NO $_3^-$	Arterial thrombosis	1.67	0.05
		Smoking	2.66	0.005
NO $_3^-$, NO $_2^-$, TAC, smoking, CRP	NT	CRP	2.74	0.004

Abbreviations: IgG aCL, anticardiolipin; IgG a β_2 GPI, beta-2-glycoprotein-I; IgG anti-HDL, anti high density lipoprotein; IgG anti-ApoA-I, anti apolipoprotein AI; NO $_2^-$, nitrite; NO $_3^-$, nitrate; TAC, total antioxidant capacity; NT, nitrotyrosine; CRP, C-reactive protein.

Relationship among variables in aPL.

In the regression model with NO $_2^-$ as the dependent variable and IgG aCL, IgG anti- β_2 GPI, IgG anti-HDL, IgG anti-ApoAI, aPTT, and DRVVT as independent variables, IgG anti- β_2 GPI showed only a negative trend with NO $_2^-$ (p=0.07) (Table 3). In the model with NO $_3^-$ as the dependent variable and IgG aCL, IgG anti- β_2 GPI, IgG anti-HDL, IgG anti-ApoAI, aPTT, and DRVVT as independent variables, negative predictors were IgG aCL (p=0.03) and DRVVT (p=0.03), and a trend was seen for IgG anti- β_2 -GPI (p=0.06; Table 3). In a further model none of the antibodies bore any relationship with NT as the dependent variable but with NO $_3^-$, NO $_2^-$, TAC, and CRP set as independent variables, NO $_2^-$ negatively predicted NT (p=0.05; Table 3).

Table 3. Predictors of nitric oxide metabolites and nitrotyrosine in the aPL group.

Variables in regression model				
Independent	Dependent	Predictors	t	p-value
Persistent carriers of antiphospholipid antibodies				
IgG aCL, IgG a β_2 GPI, IgG anti-HDL, IgG anti-ApoAI, aPTT, DRVVT	NO $_3^-$	IgG aCL	-2.06	0.03
		DRVVT	-1.93	0.03
		IgG a β_2 GPI	-1.68	0.06
NO $_3^-$, NO $_2^-$, TAC, smoking, CRP	NT	NO $_2^-$	-1.74	0.05

Abbreviations: IgG aCL, anticardiolipin; IgG a β_2 GPI, beta-2-glycoprotein-I; IgG anti-HDL, anti-high density lipoprotein; IgG anti-ApoAI, anti-apolipoprotein AI; aPTT, activated partial thrombiplastin time; DRVVT, dilute Russell viper venom time; NO $_2^-$, nitrite; NO $_3^-$, nitrate; TAC, total antioxidant capacity; NT, nitrotyrosine; CRP, C-reactive protein; SLEDAI, systemic lupus erythematosus disease activity index

Relationship among variables in IT

In the regression model with NT as the dependent variable and NO $_3^-$, NO $_2^-$, TAC, smoking, and CRP as independent variables, only CRP independently predicted NT (p=0.0006; Table 4).

Relationship among variables in SLE

In the regression model with NO $_3^-$ as the dependent variable and IgG anti- β_2 -GPI, IgG anti-HDL, IgG anti-ApoA-I, and IgG aCL as the independent variable, IgG aCL negatively predicted NO $_3^-$ (p=0.03); a similar result was obtained when NO $_2^-$ was substituted for NO $_3^-$ (p=0.02; Table 3). In the model with NT as the dependent variable and NO $_3^-$, NO $_2^-$, TAC, smoking, and CRP as independent variables, NO $_2^-$ negatively predicted NT (p=0.002) and NO $_3^-$ positively predicted NT (p=0.001; Table 4). Finally, in the model with SLEDAI as the dependent variable and NO $_3^-$, NO $_2^-$, TAC, CRP, smoking, and NT as the independent variables, NT predicted SLEDAI (p=0.009) and a trend was seen for CRP (p=0.08; Table 4).

Relationship among variables in the control group

No effect on NO_2^- was seen in a multiple regression model with NO_2^- as the dependent variable and age, sex, smoking, IgG anti-HDL, and IgG anti-ApoI as explanatory variables. In a similar model where NO_3^- was set as the dependent variable, smoking independently predicted NO_3^- ($p=0.003$; Table 4). Similarly, when NT was set as the dependent variable with age, sex, smoking, TAC, NO_2^- and NO_3^- as explanatory variables, smoking independently predicted NT ($p=0.02$) alongside NO_3^- ($p=0.04$; Table 4).

Table 4. Predictors of nitric oxide metabolites and nitrotyrosine in non aPL groups.

Variables in regression model				
Independent	Dependent	Predictors	t	p-value
Inherited thrombophilia				
NO_3^- , NO_2^- , TAC, smoking, CRP	NT	CRP	3.75	0.0006
Systemic lupus erythematosus				
IgG aCL, IgG $\alpha\beta_2\text{GPI}$, IgG aHDL, IgG aApoA-I	NO_2^-	IgG aCL	-2.12	0.02
IgG aCL, IgG $\alpha\beta_2\text{GPI}$, IgG anti-HDL, IgG anti-ApoAI	NO_3^-	IgG aCL	-1.84	0.03
NO_3^- , NO_2^- , TAC, smoking, CRP	NT	NO_2^-	-3.25	0.002
		NO_3^-	3.65	0.001
NO_3^- , NO_2^- , TAC, CRP, smoking, NT	SLEDAI	NT	2.55	0.009
		CRP	1.44	0.08
Normal controls				
Age, sex, TAC, CRP, smoking, NT	NO_3^-	Smoking	2.90	0.003
Age, sex, NO_3^- , NO_2^- , TAC, CRP, smoking	NT	Smoking	2.21	0.02
		NO_3^-	1.75	0.04

Abbreviations: IgG aCL, anticardiolipin; IgG $\alpha\beta_2\text{GPI}$, beta-2-glycoprotein-I; IgG anti-HDL, anti high density lipoprotein; IgG anti-ApoAI, anti apolipoprotein A-I; aPTT, activated partial thrombiplastin time; DRVVT, dilute Russell viper venom time; NO_2^- , nitrite; NO_3^- , nitrate; TAC, total antioxidant capacity; NT, nitrotyrosine; CRP, C-reactive protein; SLEDAI, systemic lupus erythematosus disease activity index

DISCUSSION

A small study had demonstrated lower urinary NO_2^- in PAPS, in negative correlation with serum IgG aCL titre (53). Of the NO metabolites, it is widely accepted that in humans, only NO_2^- reflects changes in eNOS activity (54,55) and endothelial dysfunction (56) known to be impaired in APS (57). To validate the clinical significance of NO_2^- and NO_3^- with regard to thrombosis in PAPS we employed as comparator patients with IT who had vascular occlusions, aPL patients who had no vascular occlusions, patients with SLE as an inflammatory disease control group, and healthy subjects. The low average concentration of NO_2^- found in PAPS and IT suggests that reduced NO_2^- may be involved in the vascular events of these patients, although causality cannot be established since vessel occlusion might have led to reduced NO_2^- : in fact, the number of vascular occlusions was a negative independent predictor of NO_2^- in PAPS. Nevertheless NO_2^- was also low in the aPL group who never had vessel occlusions, suggesting that impaired NO_2^- generation may precede and hence represent a predisposing factor for thrombosis. Reduced NO has a wider importance in the vascular biology of PAPS. NO maintains vascular homeostasis against the vasopressor effects of endothelin-1, of F2-isoprostanes derived from lipid peroxidation, and of thromboxane generated after platelet activation (58). Indeed, elevated plasma and/or urinary levels of the aforementioned molecules have all been described in PAPS (49,59,60): hence loss of the antiplatelet effect of NO may be relevant to thrombosis (61) and loss of its vasodilator effect may be relevant both to thrombosis and to atherosclerosis.

Of further interest, aPL negatively predicted NO_3^- and/or NO_2^- in the PAPS, aPL, and SLE groups, adding further to the pathogenic potential of aPL. We previously demonstrated that monoclonal IgG aCL was associated with decreased concentrations of NO metabolites in a mouse model (50). Inducible NOS may generate a 1000-fold higher concentration of NO than eNOS, which is associated with vascular damage and cytotoxic effects, whereas NO is generated by eNOS for short periods of time to maintain vascular homeostasis (62). Likely

only the latter pathway is impaired in PAPS, whereas the former pathway may be more active in SLE, the group that showed a greater concentration of NO_3^- , although a large difference was not seen because our patients with SLE were mostly clinic attendees devoid of acute or chronic renal disease with low disease activity, hence inflammatory activity. Notwithstanding, our data in SLE would be consistent with possible iNOS activation, cytotoxic release of NO ultimately leading to tyrosine nitration due to the inflammatory nature of the disease (62). Among the antibodies that might have had an influence on NO IgG anti-HDL and IgG anti-ApoAI were included because in previous work their average plasma concentrations was elevated in SLE and PAPS, where they adversely affected the antioxidant system associated with HDL, favouring oxidation (63,64). In our present study, they failed to show any relation with NO metabolites, indirectly confirming their specificity in blunting the anti-atherogenic and anti-inflammatory effects of HDL (63). To investigate nitrative stress in PAPS we measured crude plasma NT: this was higher in the SLE group, where NT related to disease activity and was predicted by NO_3^- , in keeping with findings from other inflammatory rheumatic disorders (65). On the other hand, having demonstrated that low grade inflammation characterizes PAPS (66), we found that CRP was an independent predictor of NT in the PAPS group, suggesting that nitrative stress and low grade inflammation may be related phenomena in these thrombotic patients. Interestingly, smoking predicted NO_3^- in PAPS, and it is known that active (29) and passive smoking (67) may induce oxidative stress.

Our study has several limitations: a) its retrospective design prevented a full appreciation of the role of NO in thrombosis as most PAPS patients were diagnosed after vascular occlusion; b) our SLE group comprised female patients of whom only 5 had a history of thrombosis; however, we had opted for inclusion of the SLE group mostly to show the inflammatory behaviour of NO rather than to control for thrombosis, which was provided for by the IT group; c) the method we employed for the measurement of NO metabolites is not

sensitive enough to detect nanomolar concentrations of NO_2^- and NO_3^- (68), and we did not evaluate eNOS and/or iNOS gene polymorphisms that may have accounted for differences in measured metabolites (69,70), although our groups would have been too small to yield significant data. In conclusion, our study, alongside our previous animal data (50), indicates an impairment of the vascular biology of NO in PAPS, the consequences of which may be thrombosis and atherosclerosis. With regard to the former, we cannot define whether decreased NO_2^- is a cause or an effect of previous thromboses, but the low NO_2^- in the aPL group without vessel occlusions and the relationship between NO metabolites and aPL in the PAPS, aPL, and SLE groups indicate that aPL may negatively influence some physiological activities of NO. With regard to the latter, patients with PAPS exhibit a certain degree of nitritative stress that relates to low grade inflammation, also noted in other settings (71): given the finding of NT in vessels of atherosclerotic patients (35), this aspect needs to be further explored in PAPS. From a practical point of view, our study provides evidence that smoking should be avoided in patients with PAPS.

CHAPTER VII

INFLAMMATION AND IMMUNE ACTIVATION IN THROMBOTIC PRIMARY ANTIPHOSPHOLIPID SYNDROME.

INTRODUCTION

Inflammatory and immune markers in atherosclerosis

Basic science studies have demonstrated that atherosclerosis is a chronic inflammatory process whereas epidemiological and clinical studies have shown independent relationships between plasma levels of certain inflammatory proteins such as C-reactive protein (CRP) and serum amyloid A (SAA) and cardiovascular disease. Conceivably altered plasma levels of these inflammatory proteins reflect the presence of inflammation in the arterial wall (1, 2) or elsewhere in the body that might increase the risk for atherosclerosis.

C-reactive protein

CRP is a member of the pentraxin family containing five non-covalently linked protomers surrounding a central pore. It is transported free in plasma rather than bound to circulating lipoproteins, although it can interact with oxidized phospholipids and oxidized lipoproteins in vitro (3). CRP activates complement and binds to Fc receptors, which may facilitate the uptake and clearance of apoptotic and necrotic cells during the acute-phase response (4).

CRP and prediction of clinical cardiovascular events

Being the first acute-phase protein identified CRP is the best studied marker of inflammation in humans. Increased levels of CRP predict first clinical events, recurrent events, coronary heart disease end points, and stroke (reviewed in 5). In certain studies, CRP was a more powerful predictor of cardiovascular risk than traditional risk factors such as LDL (6). The risk prediction was independent of LDL and HDL cholesterol (7) and beyond the

power derived from using Framingham risk scores (8). The magnitude of these predictive powers have been recently challenged by the Reykjavik Prospective Cohort Study (9), where multivariate analysis suggested that measuring CRP added less to the predictive power than previous studies and were less powerful than traditional risk factors. After adjustment for smoking status, body mass index, and total blood cholesterol, participants in the highest tertile of CRP had only a 1.5-fold higher risk for coronary artery disease than those in the lowest tertile. In contrast, the odds ratio was 2.4 for subjects with increased cholesterol and 1.9 for smokers. Strengths of this study included the large number of subjects (>20,000, the largest number reported to date), the large number of cardiovascular events, the length of follow-up (20 years), and low rates of dropout in the study population (9). These observations suggest that CRP might contribute less to predictions than traditional risk factors of cardiovascular disease.

CRP and atherogenesis

The atherogenic mechanisms of CRP have been investigated mostly in model systems using cultured endothelial cells. CRP is able to impair production of anti-thrombotic molecules such as nitric oxide (10) and prostacyclin (11) and increased production of potentially atherogenic molecules such as endothelin-1 (12), various cell adhesion molecules (12, 13), monocyte chemoattractant protein 1 (13), interleukin-8 (14), and PAI-1 (15). CRP induces macrophage secretion of tissue factor (16) and their production of reactive oxygen species (17) and pro-inflammatory cytokines (18). CRP also increases the uptake of oxidized LDL (3), and favors monocyte adhesion and chemotaxis (19, 20).

The elevated concentration and the purity of the CRP employed represent inherent limitations to these in vitro studies. Given that CRP has been detected immunohistochemically in atherosclerotic lesions (3, 21, 22) it appears that CRP might be involved from early to late atherosclerosis through a continuum of endothelial cell injury,

impairment of vasodilation, enhanced monocyte adhesion, lipid accumulation by monocyte-macrophages, smooth muscle cell proliferation, plaque rupture and thrombosis.

Serum amyloid A

SAA is an amphipathic, α -helical apolipoprotein that is transported in the circulation primarily in association with HDL (23-25). Plasma SAA increases rapidly during acute inflammation in humans and mice (26). Although most SAA is produced by the liver, it can be produced by extrahepatic sources: indeed messenger RNA for SAA has been detected in all of the major cell types present in atherosclerotic lesions which contain both acute-phase and constitutive forms of SAA protein (27).

SAA and prediction of clinical cardiovascular events

In several observational and prospective studies, the risk of cardiovascular disease associated with SAA paralleled that seen with CRP although the absolute level of risk was generally smaller. A chronic modest increase in SAA level also appears to be associated with an increased risk of cardiovascular disease (28-31).

SAA and atherogenesis

In vitro and in vivo experiments in animals indicate that apolipoprotein SAA is a chemoattractant for inflammatory cells such as monocytes, polymorphonuclear leukocytes, and T-lymphocytes (31, 32) involved in atherogenesis. Lipoprotein-associated SAA plays a role in cholesterol transport by increasing the delivery of cholesterol to peripheral cells (33, 34) whereas non-HDL-associated SAA promotes cholesterol efflux by both ABCA1-dependent and -independent mechanisms (35). SAA circulates in the blood bound to HDL, but it is conceivable that SAA not associated with lipoproteins could be formed from HDL in the artery wall or secreted directly by artery wall cells (27). Thus, lipoprotein-associated and non-lipoprotein-associated SAA might play different roles in the delivery and removal of

cholesterol from cells at inflamed or injured sites. SAA has been shown to displace apoA-I from HDL in vitro with functional consequences (36).

Chronic increases of SAA might induce the stimulation of monocyte adhesion and chemotaxis into the artery wall and increased delivery of cholesterol to artery wall cells, two processes that might contribute to the initiation and progression of atherosclerotic lesions. Because SAA binds to proteoglycans (37), chronic inflammation might facilitate the binding of SAA-containing HDL to extracellular vascular proteoglycans, which would favor the retention and modification of HDL by the vascular matrix preventing HDL from participating in reverse cholesterol transport and inhibiting oxidative processes in the artery wall. Moreover, modification of the lipid and protein components of trapped HDL might increase its interactions with macrophage scavenger receptors and render the lipoprotein atherogenic, similar to what happens with LDL (38). Therefore SAA may behave as a marker and a mediator of atherosclerosis.

CD14: membrane bound and soluble

The CD14 receptor is a 356 amino acid membrane glycoprotein where the C-terminal leader sequence of 28–30 amino acids is replaced by a glycosyl phosphatidylinositol (GPI) anchor after translation that maintains CD14 attached to the cellular membrane (39). Membrane CD14 (mCD14) is expressed on the surface of monocytes and the expression increases with differentiation of monocytes into macrophages. Shedding of mCD14 induced by serine proteases leads to soluble CD14 (sCD14) present in plasma as two isoforms with different molecular weights. Because mCD14 is not a trans-membrane protein a co-receptor is needed to activate the mCD14 intracellular signalling pathways. The most important of the CD14 co-receptors is the Toll like receptor 4 (TLR4) (39). The TLR4 is a receptor for lipopolysaccharide and heat-shock proteins that cannot function in isolation but co-operates with mCD14 to generate an innate immune response (40). Binding to the TLR4/mCD14

complex initiates trans-membrane signalling through to activation of nuclear factor kappa-beta, production of pro-inflammatory cytokines from monocyte/macrophages and adhesion molecules from endothelial cells (39).

CD14 and prediction of clinical cardiovascular events

Immunocytochemistry studies show that TLR4/mCD14 complex is expressed on macrophages and endothelial cells within the atherosclerotic plaque (41) but there is no relationship between plasma concentrations of sCD14 and stable coronary artery disease or carotid IMT (42-44).

Neopterin

Neopterin (NPT) is a pteridine compound deriving from guanosine triphosphate produced in large amounts by human monocytes/macrophages upon stimulation with the cytokine interferon- γ released by activated T-lymphocytes. As such it is a marker for immune system activation that is suitable for the assessment of vascular diseases such as atherosclerosis (45).

Neopterin and prediction of cardiovascular disease

Early studies revealed elevated serum NPT in subjects with different stages of peripheral vascular disease (46) and with carotid atherosclerosis (47). Having extended the studies to subjects with or without stable coronary artery disease NPT resulted predictive of total and cardiovascular mortality after adjusting for traditional risk factors for atherosclerosis (48). Moreover NPT predicted patients undergoing coronary artery disease progression (49) and identified patients at long-term risk of death or recurrent acute coronary events after acute coronary syndrome (50) and in patients with chronic stable angina pectoris (51).

Antiphospholipid syndrome, immune activation and inflammation

The presence of antibodies and T-cell clones against β_2 GPI in the peripheral blood of affected patients define PAPS as an autoimmune disorder (52, 53). Antibodies against β_2 GPI promote endothelial cell, monocyte and coagulation activation: the vessel wall shifts to a pro-adhesive and pro-thrombotic phenotype (54), monocytes express and release TNF and tissue factor (55) and thrombosis may ensue. Circulating levels of pro-inflammatory cytokines are elevated in APS (56-58) and some novel pro-inflammatory genes are up-regulated in endothelial cells stimulated with IgG β_2 GPI (59) but PAPS is not an inflammatory disorder in the traditional sense as only modest increases of CRP have been detected (60-63). Despite the presence of auto-reactive T-cell clones (52, 53) in vivo markers of immune activation have not been investigated in PAPS. Stimulated T helper cells release IFN- γ that activates monocytes with the release of NPT and modulation of their surface component CD14 (45). Therefore, NPT is a specific marker of immune cooperation, whereas soluble CD14 (sCD14) may also reflect monocyte activation in the absence of IFN- γ as it can behave as an acute-phase reactant (64). Moreover, the observation that β_2 GPI is a ligand for oxLDL (65) and that the resulting oxLDL- β_2 GPI complex binds CRP (66) prompted us to evaluate the clinical relevance of CRP, SAA (inflammatory markers), NPT and sCD14 (immune activation markers) and their relationship with oxLDL- β_2 GPI-CRP in thrombotic patients with PAPS

EXPERIMENTAL DATA

Patients

The study was conceived as a cross-sectional case double-control to serve as a baseline for an interventional trial. Consecutive thrombotic patients fulfilling recent criteria for PAPS and patients with IT attending the Coagulation Unit of the Cardarelli Hospital (Naples, Italy) were invited to participate in the study that was carried out according to the revised Declaration of

Helsinki, with approval of the Ethics Board of the Hospital and the written consent of all participants. Exclusion criteria were acute or chronic hepatic, renal, lung disease, diabetes, a recent history of acute infection (within 6 weeks), post-thrombotic syndrome with venous ulcerations, a positive urinary dipstick for nitrates on the day of sampling, treatment with steroids, statins and fibrates. Patients are seen on average every 3–4 weeks for their oral anti-coagulation monitoring and are instructed to self-report any illness they might incur during the intervening periods. Every year, their lipid profile, kidney and liver function tests are checked. Of the PAPS attendees (n=50), two were excluded because they had gradually developed AS and SLE, one had developed kidney cancer, two were pregnant, one had suffered a recent recurrent event, one had post-thrombotic syndrome and two were evasive regarding their smoking and contraceptive status. Of the IT (n=46) attendees, two were excluded for venous ulcerations in lower limbs. The study therefore included 41 thrombotic PAPS patients, 44 IT patients and 39 control subjects. The laboratory criteria for diagnosis of APS define Category IIa as lupus anti-coagulant (LA) alone, IIb as aCL alone, IIc as anti- β 2GPI alone and Category I as any combination of the previous: of our PAPS patients, eight were in laboratory Category IIa and the remaining in Category I. From the therapeutic viewpoint, all were taking warfarin, three were on carbamazepine for post-ischaemic epilepsy, two on β -blockers six on folic acid. Of the IT patients, all were on warfarin, two were on β -blockers five on folic acid and two on angiotensin-converting enzyme inhibitors. The control group included 19 blood donors, 9 healthy hospital personnel and 11 subjects on warfarin for mitral valve replacement (mitral valve prolapse, n=4; childhood rheumatic fever, n=4; ventricular septal defects with congenital mitral incompetence, n=3) that occurred on average 22 ± 8 yrs earlier. This partially accounts for the lifelong warfarin intake of the other two groups. Demographics and clinical features of all participants are shown in Table 1.

Table 1. Demographics of participants.

	Controls			
	Healthy	Warfarin	IT	PAPS
No	28	11	44	41
Age (mean±SD)	37±9	60±10	51±14	40±12
M/F	10/18	4/7	19/25	18/23
Age at thrombosis (mean±SD)			39±14	35±11
<i>Thrombosis type</i>				
Arterial			9	13
Venous			32	24
Arterial+ venous			3	4
Thrombosis number				
1			31	13
2			10	14
3 or >3			3	4
IgG aCL (mean±SD)	11±12	6.3±1.5	7.5±6.8	131±143
IgG β_2 GPI (mean±SD)	2.3±1.0	3.1±0.07	4.2±0.8	119±67
<i>Smoking (sig/day)</i>				
<11	6	2	5	3
11-20	1	0	2	3
>20	1	1	3	2
Ex smoker	0	0	0	4
Tryglicerides >150 mg/dl	1	2	3	4
Cholesterol >200 mg/dl	3	4	5	4
BMI >30	0	0	1	2
HRT	1	1	2	0
OC	5	0	2	3
Menopause	1	3	6	2

IT: inherited thrombophilia; PAPS: primary antiphospholipid syndrome; aCL: anticardiolipin; β_2 GPI: beta-2 glycoprotein I; BMI: body mass index; HRT: hormone replacement treatment; OC: oral contraception.

RESULTS

Comparison of inflammatory and immune markers across groups

Average plasma levels of CRP, SAA, NPT, sCD14, CRP/oxLDL/ β_2 GPI and oxLDL/ β_2 GPI were higher in PAPS than other groups considered (Figure 1). Amongst age, gender, menopause, oral contraception, smoking and obesity, ANCOVA identified only age as a significant confounder for NPT ($p=0.007$), sCD14 ($p=0.0001$), oxLDL/ β_2 GPI ($p=0.02$) and SAA ($p=0.04$). Age-adjusted significances between groups were $p<0.01$ for SAA, $p<0.0001$ for NPT, $p=0.007$ for sCD14, $p=0.0001$ for oxLDL/ β_2 GPI. Within PAPS, IT and CTR, the proportion of participants positive for CRP was 31, 13 and 10%, respectively ($p=0.02$), for SAA 31, 6 and 2%, respectively ($p<0.0001$), for NPT 68, 27 and 15%, respectively ($p<0.0001$), for sCD14 48, 34 and 23%, respectively ($p=0.054$), for CRP/oxLDL/ β_2 GPI 53, 31 and 18%, respectively ($p<0.003$), oxLDL/ β_2 GPI 41, 9 and 10%, respectively ($p=0.0002$). Sensitivity and specificity of CRP/oxLDL/ β_2 GPI was 51 and 100%, respectively with regards to thrombotic PAPS, but 53 and 64%, respectively with regards to arterial thrombosis

Relationship amongst variables in the PAPS group

Two regression models were employed: the first assessed the predictive effect of IgG aCL, IgG β_2 GPI, gender, event type, event number, age at event and smoking status on plasma levels of CRP, SAA, NPT, sCD14, CRP/oxLDL/ β_2 GPI and oxLDL/ β_2 GPI; the second assessed the relationship between immune activation markers and inflammatory markers after correction for gender, event type, event number, age at event, smoking status in the absence of IgG aCL and IgG β_2 GPI. Remarkably, in the first model, IgG β_2 GPI was an independent predictor of SAA, NPT, sCD14 and CRP/oxLDL/ β_2 GPI. The number of thrombotic events predicted SAA and NPT, whereas arterial thrombosis predicted NPT and sCD14 (Table 2).

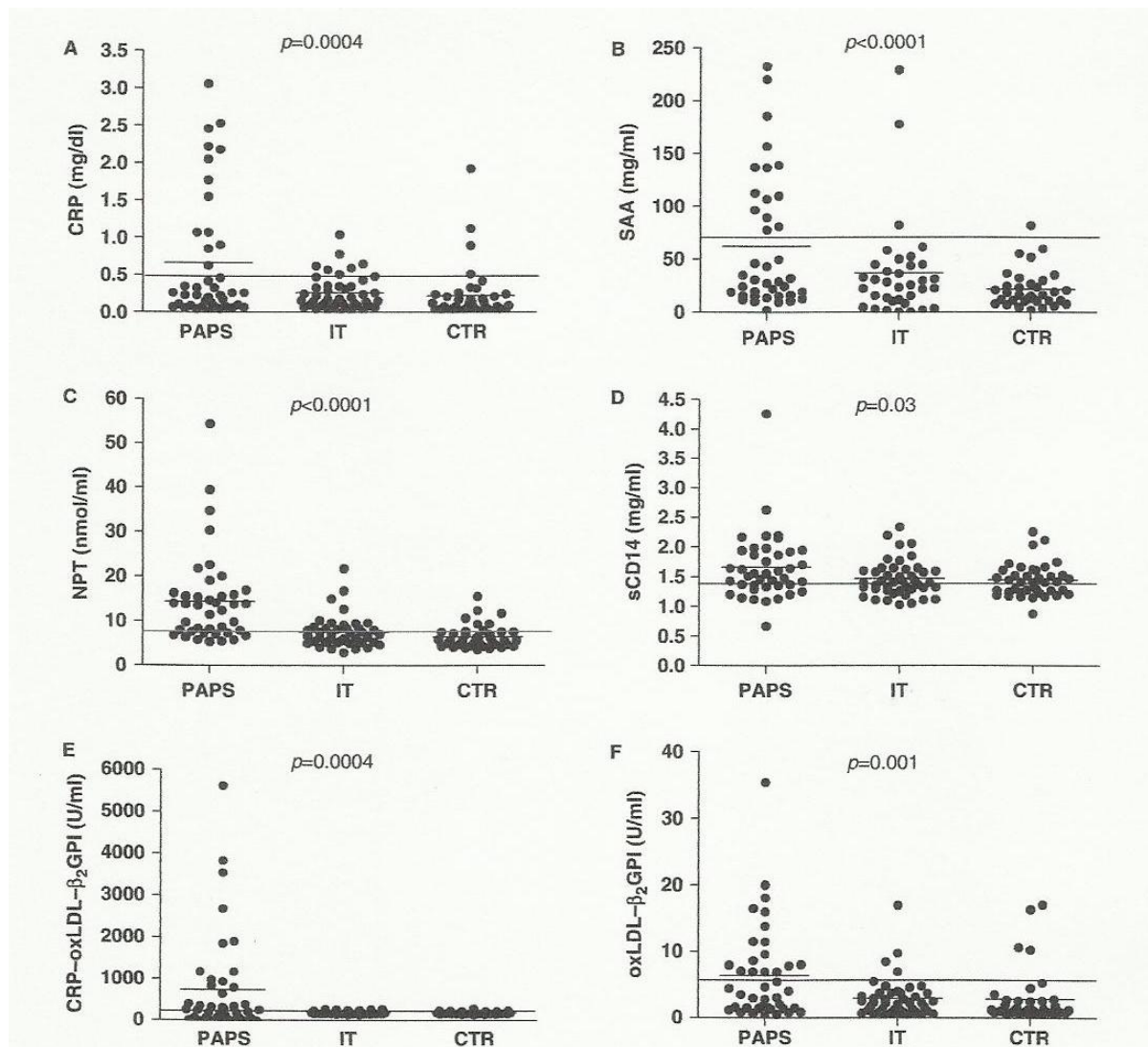


Figure 1. Average levels of (A) hs-CRP, (B) SAA, (C) NPT, (D) sCD14, (E) CRP bound to the oxLDL- β_2 GPI and (F) oxLDL- β_2 GPI complex in PAPS, IT and CTR. The long horizontal bar is the cut-off level for each variable arbitrarily set at the mean \pm 5 SEM. The P-value represents the overall ANOVA significance. Bonferroni's multiple comparison revealed: for hs-CRP, PAPS vs IT and PAPS vs CTR, both $p<0.01$; for SAA, PAPS vs CTR, $p<0.01$; for NPT, PAPS vs IT and PAPS vs CTR, both $p<0.001$; for CRP-oxLDL- β_2 GPI, PAPS vs IT and PAPS vs CTR, both $p<0.01$; for oxLDL β_2 GPI PAPS vs IT and PAPS vs CTR, both $p<0.01$. No post hoc values were derived for sCD14.

Table 2. Independent predictors of inflammatory and immune activation markers in PAPS including IgG aCL and IgG β_2 GPI as independent variables.

Dependent variable	Predictors	β	p-value
SAA	Thrombosis number	0.572	0.003
	IgG β_2 GPI	0.424	0.06
NPT	Arterial thrombosis	0.405	0.03
	Thrombosis number	0.390	0.04
	IgG β_2 GPI	0.477	0.04
sCD14	IgG β_2 GPI	0.764	0.0001
	Age	0.993	0.001
	Arterial thrombosis	0.395	0.01
CRP-oxLDL- β_2 GPI	IgG β_2 GPI	0.574	0.02
oxLDL- β_2 GPI	IgG aCL	0.714	0.0001

Independent variables in the model were: age, sex, thrombosis type (arterial, venous), thrombosis number, age at thrombosis, smoking status, IgG β_2 GPI, IgG aCL.

Indeed, the average plasma concentration of NPT was greater in patients with arterial than venous thrombosis (16.7 ± 2.4 vs 11 ± 0.9 nmol/ml, $p=0.02$) but that of sCD14 did not reach significance (data not shown). On the other hand, average plasma concentration of SAA was almost 50% higher in patients with arterial than venous thrombosis (85 ± 18 vs 45 ± 9 mg/ml, $p=0.04$). In the second regression model, NPT and sCD14 correlated with each other as did CRP/oxLDL/ β_2 GPI and SAA (Table 3).

Table 3. Independent predictors of inflammatory and immune activation markers in PAPS excluding IgG aCL and IgG β_2 GPI as independent variables

Dependent variable	Predictors	β	p-value
SAA	CRP-oxLDL- β_2 GPI	0.642	0.002
NPT	sCD14	0.723	0.002
sCD14	NPT	0.709	0.002
	Age	0.764	0.025
CRP-oxLDL- β_2 GPI	SAA	0.787	0.002

Independent variables in the model were: age, sex, thrombosis type (arterial, venous), thrombosis number, age at thrombosis, smoking status, CRP, SAA, NPT, sCD14, and CRP-oxLDL- β_2 GPI: each of the dependent variables was investigated omitting it from the independent variable list.

Relationship amongst variables in the IT group

Multiple regression analysis strongly revealed interrelations between SAA and CRP as well as between NPT and sCD14 (Table 4).

Table 4. Independent predictors of inflammatory and immune activation markers in inherited thrombophilia

Dependent variable	Predictors	β	p-value
CRP	SAA	0.547	0.009
SAA	CRP	0.518	0.009
	sCD14	0.447	0.07
NPT	sCD14	0.685	0.0001

Independent variables in the model were: age, sex, thrombosis type (arterial, venous), thrombosis number, age at thrombosis, smoking status, CRP, SAA, NPT, sCD14, and CRP-oxLDL- β_2 GPI.

Relationship amongst variables in the control group

Multiple regression analysis identified oxLDL/ β_2 GPI as an independent predictor of CRP, SAA and sCD14, whereas the inflammatory markers were strongly related (Table 5).

Table 5 Independent predictors of inflammatory and immune activation markers in normal controls

Dependent variable	Predictors	β	p-value
CRP	oxLDL- β_2 GPI	1.039	0.0001
	SAA	-0.276	0.03
SAA	oxLDL- β_2 GPI	1.118	0.0001
	NPT	0.310	0.03
sCD14	oxLDL- β_2 GPI	0.934	0.008
NPT	SAA	0.685	0.0001

Independent variables in the model were: age, sex, thrombosis type (arterial, venous), thrombosis number, age at thrombosis, smoking status, CRP, SAA, NPT, sCD14, and CRP-oxLDL- β_2 GPI: each of the dependent variables was investigated omitting it from the independent variable list.

DISCUSSION

CRP and SAA are acute-phase plasma proteins principally expressed and induced in the liver under the regulation of the pro-inflammatory cytokines IL-1, IL-6 and TNF- α though non-acute-phase elevations may occur in several chronic inflammatory diseases such as RA and atherosclerosis (67). The current survey reveals that a significant number of thrombotic PAPS patients display a low-grade inflammatory state that must be due to the disease itself, as IT patients did not show similar elevations of CRP and SAA. Thus, we support a relationship independent of thrombosis between LA and the inflammatory markers, CRP (62), factor VIII and fibrinogen (61); elevated levels of CRP independently predicted residual or recurrent symptoms in a cohort of aPL-positive patients with neurological manifestations, mostly cerebral infarctions and transient ischaemic attacks (63).

Contrary to the lack of clinical relevance of SAA in a very small PAPS group (68), SAA was elevated in our patients particularly in relation to multiple thrombotic events. Unfortunately, the cross-sectional nature of the study cannot define whether elevated SAA and CRP are cause or effect of previous occlusive events since both molecules are involved in the expression of tissue factor on monocytes and endothelial cells (69, 70). Therefore, their value as thrombotic risk markers in PAPS may only be assessed with regards to re-thrombosis as most PAPS patients will be on oral anti-coagulation after their first occlusive event.

Compared with IT and controls, our PAPS patients displayed higher plasma concentrations of NPT and of sCD14 that correlated strongly in both patient groups. In PAPS, NPT was higher in patients with multiple thromboses and both markers related to arterial occlusions: monocytes are more likely to be involved in the pathogenesis of arterial than venous thrombosis (71). The independent predictive power of IgG β_2 GPI vs sCD14 and NPT reflects the in vitro work describing monocyte activation including tissue factor expression by IgG β_2 GPI (55, 72) that adds to the T-helper activation pathway.

In a similar context, monocytes may differentiate into foam cells after the uptake of an immune complex consisting of β_2 GPI, oxLDL and a monoclonal aPL (72). Though oxLDL/ β_2 GPI complex is formed as an attempt by β_2 GPI to prevent LDL oxidation and its uptake by monocyte scavenger receptors, it is recognized by specific antibodies and up-taken by monocytes via Fc receptors (73). Monocytes play a pivotal role in APS: the direct effect of IgG β_2 GPI promotes tissue factor expression (55, 72) and hence clotting in the bloodstream, whereas phagocytosis of oxLDL- β_2 GPI bound to their specific antibody probably favours atherosclerosis (74).

OxLDL/ β_2 GPI complexes are found in primary and secondary APS, SLE (74) and chronic nephritis (75), disorders characterized by enhanced oxidative stress (76, 77). In PAPS, we and others had noted a correlation between plasma IgG aCL and isoprostanes, sensitive markers of

in vivo oxidative stress (78, 79), and in the light of the above this could explain the relationship between IgG aCL and oxLDL/ β_2 GPI in the present PAPS population.

Experimental work reveals that in analogy with certain natural antibodies and scavenger receptors, CRP binds phosphorylcholine (PC) moieties on apoptotic cells and on oxLDL in what appears a very primitive form of innate immunity (65). More recent in vitro data demonstrate that over a 24-h incubation period CRP can bind oxLDL/ β_2 GPI via PC in a ternary complex (58). Given the very short half-life (10 min) oxLDL when injected into mice and the co-localization of CRP, oxLDL and β_2 GPI with foamy macrophages in carotid artery plaques CRP/oxLDL/ β_2 GPI would fulfill the requirements for a specific marker of atherosclerosis (58), a hot topic in APS. In this study CRP/oxLDL/ β_2 GPI was 100% specific for PAPS but did not differentiate arterial and venous thrombosis. Interestingly, higher CRP/oxLDL/ β_2 GPI was predicted by elevated IgG β_2 GPI, indicating that an enhanced level of LDL oxidation requires buffering from both β_2 GPI and CRP. LDL oxidation is prevented by paraoxonase (PON), the enzyme that accounts for most of the anti-oxidant activity of HDL: in PAPS, decreased PON activity associates with elevated IgG β_2 GPI titres and a monoclonal aPL inhibited PON activity (PONa) in vitro (80). On the other hand, β_2 GPI buffering of oxLDL would lead to exposure of cryptic auto-antigenic epitopes on β_2 GPI with persisting antibody stimulation (81). In the case of the oxLDL/ β_2 GPI complex, the antibody response would be mostly IgG aCL; once the CRP/oxLDL/ β_2 GPI is formed, the highly specific IgG β_2 GPI would be stimulated.

In the control group, no patient had elevated CRP/oxLDL/ β_2 GPI, but some had elevated oxLDL/ β_2 GPI that was an independent predictor of CRP, SAA and sCD14: a possibility is that CRP/oxLDL/ β_2 GPI and oxLDL/ β_2 GPI are formed at high and low LDL oxidation levels, as would occur in PAPS and normal subjects, respectively. In the latter case, only β_2 GPI is

required to buffer oxLDL though the resulting oxLDL/ β_2 GPI complex still associates with CRP.

In conclusion, immune activation and low-grade inflammation occur in PAPS. The former was not unexpected but the latter raises further issues: are the inflammatory markers measured of liver or vascular origin? Endothelial cells, smooth muscle cells and monocytes can produce CRP as well as SAA in atherosclerotic lesions (27, 82, 83). Indeed, CRP/oxLDL/ β_2 GPI related to SAA in this survey. Outside the acute setting, mild plasma elevations of CRP, SAA and NPT represent an inflammatory signature of advanced atherosclerosis (84, 85) but do not add discriminatory diagnostic power over established cardiovascular risk factors (30). The picture may be different in PAPS where atherosclerosis may not necessarily involve traditional cardiovascular risk factors (86): as number and type of events were linked to plasma levels of CRP, SAA and NPT these may represent useful markers alongside CRP/oxLDL/ β_2 GPI to predict vascular disease progression and to monitor monocyte and vascular activity in interventional trials (87-89).

CHAPTER VIII

ANTIPHOSHOLIPID ANTIBODIES, HAEMOSTATIC VARIABLES AND ATHEROSCLEROSIS IN THROMBOTIC PRIMARY ANTIPHOSPHOLIPID SYNDROME

INTRODUCTION

Antiphospholipid antibodies, arterial disease and atherosclerosis

The issue of arterial disease and atherosclerosis is at the forefront of clinical research in the APS. Amongst non-traditional risk factors for atherosclerosis aPL antibodies partially explain, alongside traditional risk factors (1), the elevated risk of cardiovascular disease in SLE (reviewed in 2). Before PAPS became a distinct clinical entity, the observation that some cases of vascular occlusion in APS were due to a proliferative vasculopathy rather than acute thrombosis (2, 3) lead to the search for an association between arterial disease and aPL. Early studies focused essentially on aCL, others on the LA. A cross-sectional survey in 234 patients who underwent vascular surgery for peripheral arterial disease revealed a positive aPL test in 25% of patients. These patients were 1.8-times more likely to have had previous lower-limb vascular surgery, 5.6-times more likely to have had occlusion of that procedure and four-times more likely to be female (4). The same authors later reported that aPL-positive (aCL and/or LA) patients undergoing lower-limb arterial bypass surgery were more likely to have progression of their disease post-bypass compared with aPL-negative patients (73 vs 37%; $p < 0.0001$). aPL also appeared superiorly predictive of arterial disease progression compared with traditional risk factors (5). A case-control study in 62 patients younger than 50 years of age operated for atherosclerotic peripheral vascular disease detected IgG aCL in 14.5% of the patients. Interestingly, patients with IgG aCL were mostly female and had a lower prevalence of hyperlipidemia/dyslipidemia compared with patients without IgG aCLs (6). In a cohort of 123 patients with a variety of vascular involvement IgG aCL were more common in patients

with cerebral and peripheral involvement (7). With regards to the LA, the dilute Russell's viper venom time (DRVVT) and its platelet neutralization procedure was found in 43% of 60 surgical vascular patients and in none of the 20 general surgical controls ($p = 0.0002$) (8) though a later survey in 150 patients with peripheral vascular disease found a lower 4% prevalence (9).

Other aPL directed against β_2 GPI complexed with oxLDL and β_2 GPI complexed with 7-ketocholesteryl-9-carboxynonanoate (oxLig-1), the very oxLDL-derived moiety that binds β_2 GPI (10) are associated with arterial thrombosis in APS (11). Macrophages uptake β_2 GPI-oxLDL much faster in the presence of an antibody towards oxLDL- β_2 GPI (12) and there is a possibility that antibodies against β_2 GPI-oxLig-1 and β_2 GPI-oxLDL have atherogenic potential in primary APS.

Measurement of the IMT of carotid arteries via high-resolution ultrasonography of carotid is a surrogate but established method to identify at-risk patients very early on (13) This methodology was applied to 45 PAPS patients with deep vein thrombosis and to a similar number of non-APS thrombotic controls matched by age, sex and other vascular risk factors. Having scanned the carotids, the abdominal aorta, and the femoral arteries, IMT and plaque prevalence were similar in the two groups and the authors concluded that atherosclerosis was not a feature of their PAPS cohort (14). Traditional and non traditional risk factors for atherosclerosis such as plasma FNG, vWF) homocysteine (HC) and novel aPL have not been explored in atherosclerosis related PAPS.

Haemostatic variables, homocysteine, paraoxonase and atherosclerosis

Numerous prospective epidemiological studies and clinical observations have demonstrated that plasma FNG is a strong, consistent and independent cardiovascular risk factor. A modest 10% increase in plasma FNG predicts future coronary heart disease endpoints with an odds ratio of 1.8 (95% CI, 1.6 to 2.00) when the top tertile of the FNG

distribution was compared to the bottom tertile (15). On the other hand, in adults without clinically overt atherosclerotic disease elevated plasma FNG was related to carotid IMT independently of age, body mass index, arterial systolic pressure, cholesterol, LDL-cholesterol, smoking, CRP and vWF (16). To a lesser extent also PAI is a correlate of IMT (17).

Homocysteine (HC) is a sulfur-containing amino acid, rapidly oxidized in plasma to the disulfides homocystine and cysteine-homocysteine. HC is not obtained from the diet but it is synthesized from methionine via a multi-step process. First, methionine receives an adenosine group from ATP, a reaction catalyzed by S-adenosyl-methionine synthetase, to give S-adenosyl methionine (SAM). SAM then transfers the methyl group to an acceptor molecule, while the adenosine is then hydrolyzed to yield HC. HC is either converted via tetrahydrofolate (THF) back into methionine or converted to cysteine. Normal fasting plasma HC concentrations range between 5 and 15 $\mu\text{mol/L}$. Concentrations between 16-30, between 31-100, and >100 $\mu\text{mol/L}$ define moderate, intermediate and severe hyperhomocyst(e)inemia.

Homocystinuria due to defective activity of cystathionine β -synthase is a rare autosomal recessive genetic disorder (1:200000 births) leading to severe hyperhomocyst(e)inemia and a variety of abnormalities, including a high incidence of vascular pathology that may result in early death from myocardial infarction, stroke, or pulmonary embolism. Homocystinuria secondary to a heat-labile variant of MTHFR, due to a cytosine to thymine (C to T) transition at nucleotide 677 is more common (prevalence). A wealth of studies could not agree on the association between elevated HC and coronary artery disease, ischaemic stroke and peripheral vascular disease (18). Nevertheless, the Atherosclerosis Risk In Communities study detected a relationship between elevated HC and IMT of carotid arteries in asymptomatic subjects (19).

Paraoxonase-1 (PON1) is a protein of 354 amino acids with a molecular mass of 43 kDa synthesized by the liver and circulating in plasma attached to HDL. PON1 accounts for most

of the antioxidant effect of HDL against oxidation of LDL and partly explains the protective effect of HDL against atherosclerosis. PON1 deficient mice on a high-fat diet develop atherosclerotic lesions in the proximal aorta twice as large than normal mice whereas over-expression of human PON1 confers significant protection against the development of atherosclerosis in the same rodent. Moreover, signs of oxidative stress, vascular inflammation and thrombotic tendencies have been observed in PON1-deficient mice. In human studies, higher PON1 activity is associated with a lower incidence of major cardiovascular events. However, it remains uncertain whether this relationship is causal or correlational (reviewed in 20).

Herein we present two studies that explored the relation of haemostatic variables, HC and aPL with the IMT of carotid arteries and a third study comparing IMT in thrombotic PAPS with a second control group of patients with vascular occlusions due to IT to match the persistence of a thrombotic risk factor and the indefinite warfarin thrombo-prophylaxis of patients with PAPS.

EXPERIMENTAL DATA

1) IgG aCL, HAEMOSTATIC VARIABLES AND INTIMA MEDIA THICKNESS

Patients and methods: this cross-sectional study was carried out on 42 (13 male, 29 female) patients with idiopathic aPL (mean age 31 ± 10 years), 29 of whom had PAPS and 13 of whom were persistently positive for aPL in the absence of any underlying disorder. All participants were evaluated for a history of thrombosis, for coagulation anomalies suggestive of a LA and for a possible false positive venereal disease research laboratory (VDRL). None of the patients had suffered infective episodes during the study and a minimum of 9 months had elapsed from the last occlusive event in thrombotic patients. This was to allow for the acute phase nature of some of the plasma variables measured. A systolic blood pressure $>140\text{mmHg}$ and/or a diastolic blood pressure $>90\text{mmHg}$ was indicative of arterial

hypertension (or borderline hypertension). None of the participants in the study had systolic or diastolic hypertension. A total cholesterol level higher than 200mg/dl for subjects aged >29 years, higher than 225mg/dl for subjects aged 30–39 years, higher than 245mg/dl for subjects 40–49 years higher than 265mg/dl for subjects >50 years of age was indicative of hypercholesterolaemia. By this definition, only one patient (aged 28 years) had a slightly higher cholesterol level than expected for age (205 mg/dl). Hypertriglyceridaemia was defined as triglyceride level higher than 160mg/dl. Six patients had a triglyceride level higher than 160mg/dl (182 ± 16 mg=dl, range 162-203 mg/dl). None of the patients had diabetes mellitus. Demographics of the participants in the study are shown in Table 1.

Table 1. Clinical characteristics of aPL participants.

	aPL (n=42)	
	N	%
Current smokers	2	4.7
Ex-smokers	4	9.5
Menopausal	3	7.1
Oral contraceptive	2	4.7
Hypercholesterolaemia	1	2.3
Hypertriglyceridaemia	6	14.2
Lupus anticoagulant	39	92
IgG aCL (<20 GPL)	3	7.1
IgG aCL (20-80 GPL)	17	40
IgG (>80GPL)	22	52.3
Platelets >150 x 10 ⁹ /L	33	78.5
Platelets <150 x 10 ⁹ /L	5	11.9
Platelets <100 x 10 ⁹ /L	3	7.1
Platelets < 50 x 10 ⁹ /L	1	2.3
Non thrombotic	13	30.9
Arterial thrombosis	5	11.9
Venous thrombosis	21	50
Arterial + venous	3	7.1
Warfarin	25	59.5
Aspirin	8*	19

aPL: antiphospholipid antibody; aCL: anticardiolipin;

* includes four non thrombotic participants.

RESULTS

IMT and plasma variables according to gender and thrombotic status

Table 2 shows the means of variables measured in the total aPL population and then according to gender. Mean IMT was 0.44 ± 0.01 mm in the common carotid artery, 0.48 ± 0.02 mm in the carotid bifurcation and 0.41 ± 0.01 mm in the internal carotid. Males showed thicker intima-media of the internal carotid than females as well as higher mean IgG aCL ($p=0.02$) (Table 2). Patients with a history of thrombosis ($n=29$) had higher mean levels of platelets (235 ± 12 vs 143 ± 18 , $p<0.0001$), FNG (334 ± 9 vs 282 ± 13 mg=dl, $p=0.003$) and triglycerides (125 ± 8 vs 88 ± 8 mg=dl, $p=0.006$) than patients without such a history. Likewise, mean IMT of the carotid bifurcation was thicker in the thrombotic than non-thrombotic group ($0.50 \pm \pm 0.02$ vs 0.42 ± 0.02 mm, $p=0.04$) and an almost significant difference was noted for the internal carotid (0.44 ± 0.02 vs 0.37 ± 0.02 mm, $p=0.056$).

Table 2. Average concentration of measured variables according to gender.

	Total (n=42)	Male (n=13)	Female (n=29)	p-value (M vs F)	Normal Ranges
Age (years \pm SD)	36 \pm 10	38 \pm 9	35 \pm 10		
IgG aCL (GPL)	122 \pm 17	184 \pm 31	95 \pm 19	$p=0.02$	<5
IgM aCL (MPL)	41 \pm 20	20 \pm 4.2	50 \pm 29		<5
PLT (10^9 /L)	207 \pm 12	228 \pm 20	197 \pm 15		130-180
HC μ mol/L	13 \pm 1.8	16 \pm 1.6	11 \pm 2.5		<12
FNG (mg/dl)	318 \pm 8.3	329 \pm 17	313 \pm 5		160-360
VWF (IU/dl)	119 \pm 4.3	119 \pm 7	119 \pm 5		50-150
PAI (ng/ml)	39 \pm 3.4	48 \pm 8	35 \pm 3	$p=0.04$	<50
CHO (mg/dl)	181 \pm 5.1	182 \pm 9.7	180 \pm 6.2		<265
HDL (mg/dl)	45.8 \pm 1.4	43 \pm 3	46 \pm 1.5		30-70
LDL (mg/dl)	109.4 \pm 9	110 \pm 9	109 \pm 5		50-190
TG (mg/dl)	113.6 \pm 6.6	125 \pm 12	108 \pm 8		<160
IMT IC (mm)	0.41 \pm 0.01	0.48 \pm 0.03	0.39 \pm 0.01	$p=0.02$	

ACL: anticardiolipin; PLT: platelets; HC: homocysteine; FNG: fibrinogen; VWF: von Willebrand factor; PAI: plasminogen activator inhibitor; CHO: cholesterol; HDL: high density lipoprotein; LDL: low density lipoprotein; TG: triglycerides; IMT: intima media thickness; IC: internal carotid.

Relationship between plasma variables and IMT

The univariate relationship between plasma variables, age and number of thrombotic events with IMT of different carotid segments was assessed by simple regression (Table 3).

Table 3. Correlates of intima media thickness (univariate analysis).

	Common carotid		Carotid bifurcation		Internal carotid	
	R2	p-value	R2	p-value	R2	p-value
Age	0.32	<0.0001	0.21	0.002	0.22	0.002
IgG aCL	0.23	0.001	0.30	0.0002	0.40	0.0001
FNG	0.17	0.006	0.31	0.0001	0.25	0.0008
LDL	0.15	0.01				
CHO	0.13	0.02	0.14	0.01		
PAI	0.11	0.02			0.22	0.002
HC	0.09	0.04	0.16	0.009	0.15	0.01
VWF			0.13	0.01		
No Thr			0.11	0.03		

To test which factors independently predicted IMT of carotid artery segments those variables related to IMT in the univariate analysis were entered in a stepwise multiple regression model. This analysis confirmed IgG aCL, HC and FNG as independent predictors of IMT, with differing strength according to the carotid segment under study (Table 4).

Table 4. Predictors of intima media thickness (multivariate analysis).

Dependent variable	Predictor	β	p-value
Common carotid	IgG aCL	0.375	<0.005
Carotid bifurcation	FNG	0.441	0.001
	HC	0.404	<0.0001
	IgG aCL	0.337	0.008
Internal carotid	IgG aCL	0.502	<0.0001
	HC	0.405	<0.0001
	FNG	0.268	0.03

2) ANTI β_2 GPI/oxLDL, ANTI β_2 GPI-oxLig-1 AND INTIMA MEDIA THICKNESS OF CAROTID ARTERIES

Patients and methods: the study populations were drawn from consecutive patients attending the Coagulation Unit of the Cardarelli Hospital in Naples (Italy) who gave informed consent before entering the study, carried out according to the revised Declaration of Helsinki. All APS patients met the Sapporo Criteria for PAPS (Wilson et al. 1999). They were evaluated for a thrombotic event and were found to be positive for LA and aCL antibodies on two separate occasions 6 weeks apart. Patients with idiopathic aPL were detected because of the presence of a LA on routine clotting screen for minor surgical procedures or for health checks and never suffered any vascular manifestation of APS.

Table 5 Demographics of study patients.

	CTR (n=23)	IT (n=17)	aPL (n=10)	PAPS (n=29)
Female/male	13/7	11/6	7/3	20/9
Age (median, range)	35 (20-68)	40 (30-58)	40 (29-54)	38 (27-63)
IgG aCL (median, range)			59 (16-309)	118 (24-573)
IgG β_2 GPI (median, range)			147 (40-216)	183 (1-226)
FVL	0	12	0	2
PT20210	1	2	1	1
PC deficiency	0	3	0	0
IS		2		8
MI		0		1
DVT		11		15
PE		4		5
Smoking	3	4	2	5
Hypertension	0	0	2	5
Diabetes	0	0	0	1
High CHO	0	0	0	1
High TG	0	0	1	2
Aspirin	0	1	6	0
Warfarin	0	17	0	29

Seventeen patients with IT who underwent vascular occlusions served as a thrombotic control group. Vascular events were diagnosed by MRI, doppler ultrasound, ECG and echocardiogram according to vascular bed involved. Twenty-three healthy hospital personnel served as a normal control group. None of the participants were on lipid lowering agents or antioxidant drugs at the time of the study. Plasma from patients and controls were kept at 80°C until analysis. Their demographics and clinical features are presented in Table 5.

RESULTS

PON1 activity, β_2 GPI-oxLDL complexes, anti- β_2 GPI-oxLDL and anti- β_2 GPI-oxLig-1 in study groups

PON1 activity (nmol/ml/min) was relatively similar across groups, being 0.96 ± 0.25 in normal controls, 0.90 ± 0.34 in thrombotic controls, 0.87 ± 0.40 in idiopathic aPL and 0.78 ± 0.35 in thrombotic PAPS patients. Plasma levels of β_2 GPI-oxLDL complexes were highest in the control group (Figure 1A), whereas, those of IgG anti- β_2 GPI-oxLDL (Figure 1B) and anti- β_2 GPI-oxLig-1 (Figure 1C) were highest in the PAPS and aPL groups. These variables showed no gender differences in any of the groups, and no differences were noted between patients with arterial or venous thrombosis in the PAPS group.

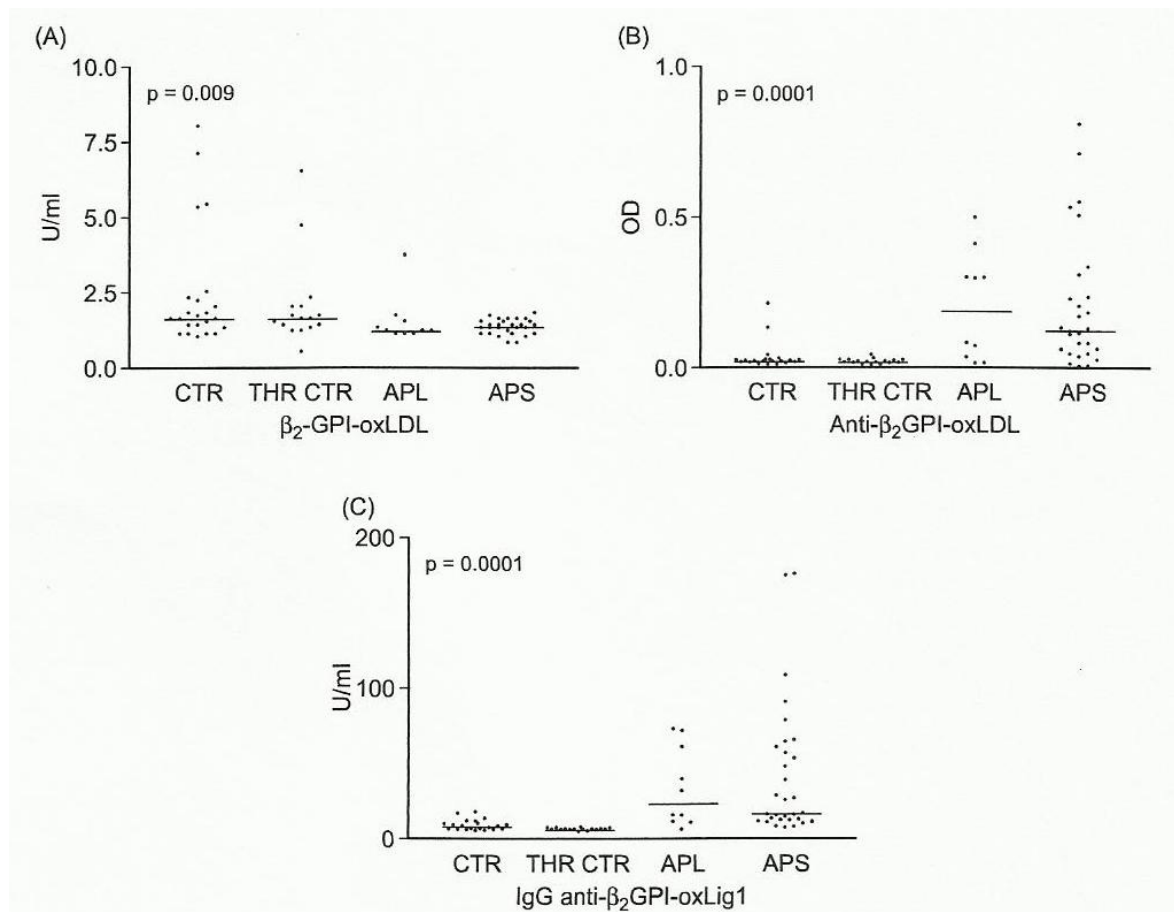


Figure 1. Median plasma levels of β_2 GPI-oxLDL (A), of IgG anti- β_2 GPI-oxLDL (B) and IgG anti- β_2 GPI-oxLig-1 (C) in the four study groups. CTR: normal controls; THR CTR: thrombotic controls; aPL: non thrombotic antiphospholipid positive subjects; APS: thrombotic antiphospholipid positive participants.

Relationship amongst variables in the PAPS group

Because two patients in this group did not have all variables measured, resulting analysis and data refer to 28 patients. With regards to antibody subtypes IgG anti- β_2 GPI-oxLig-1 positively correlated to IgG anti- β_2 GPI ($r = 0.49$, $p = 0.007$) and to IgG aCL ($r = 0.61$, $p = 0.0005$). In decreasing order of strength PONa inversely correlated to IgG anti- β_2 GPI-oxLig-1 ($r = -0.51$, $p = 0.004$), to IgG aCL ($r = -0.53$, $p = 0.003$) and to IgG anti- β_2 GPI ($r = -0.41$, $p = 0.02$). Of these, only IgG anti- β_2 GPI-oxLig-1 independently predicted PONa ($\beta = 0.44$, $p = 0.02$) in a multiple regression that took into account the effect of age and sex. In addition, PONa inversely correlated to the IMT of the IC (Figure 2A) and of the B (Figure

2B). IgG anti- β_2 GPI-oxLig-1 positively correlated to IMT of all carotid segments (Figure 3A-C). In a multiple regression model that included IgG anti- β_2 GPI-oxLig-1, IgG anti- β_2 GPI, and IgG anti- β_2 GPI-oxLDL as independent variables and IMT as a dependent variable and after correction for age and sex, IgG anti- β_2 GPI-oxLig-1 independently predicted IMT of B ($\beta = 0.40$, $p = 0.01$), CC ($\beta = 0.36$, $p = 0.02$) and IC ($\beta = 0.55$, $p = 0.003$).

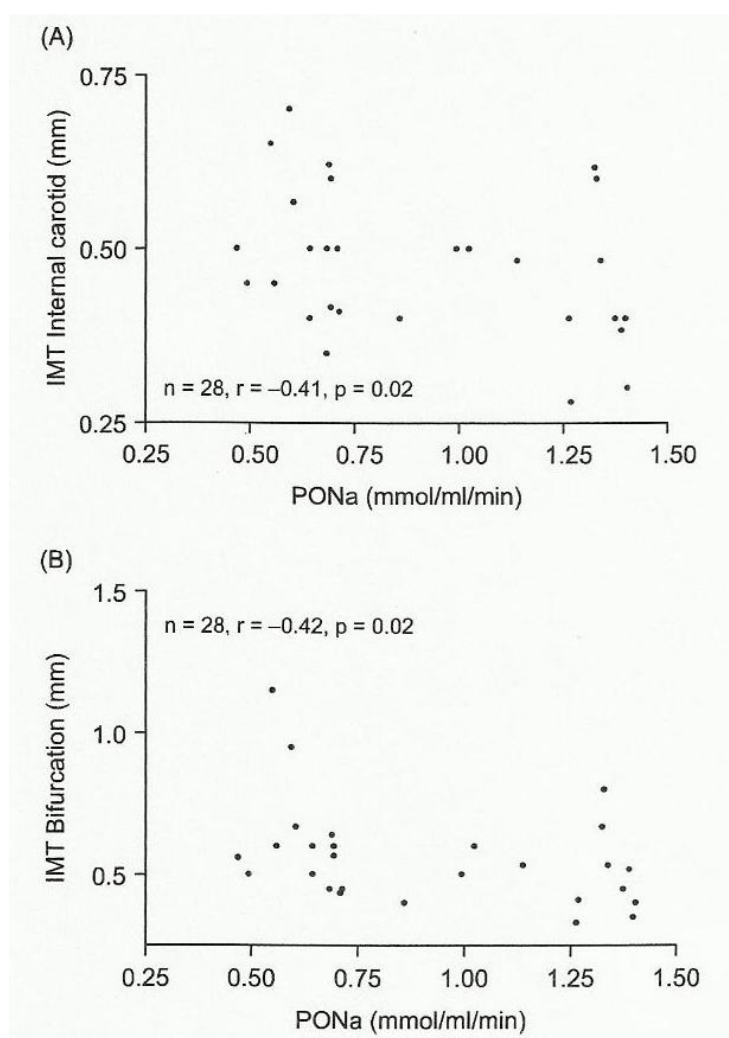


Figure 2. Relationship between paraoxonase activity (PONa) and intima media thickness (IMT) of internal carotid (A) and carotid bifurcation (B).

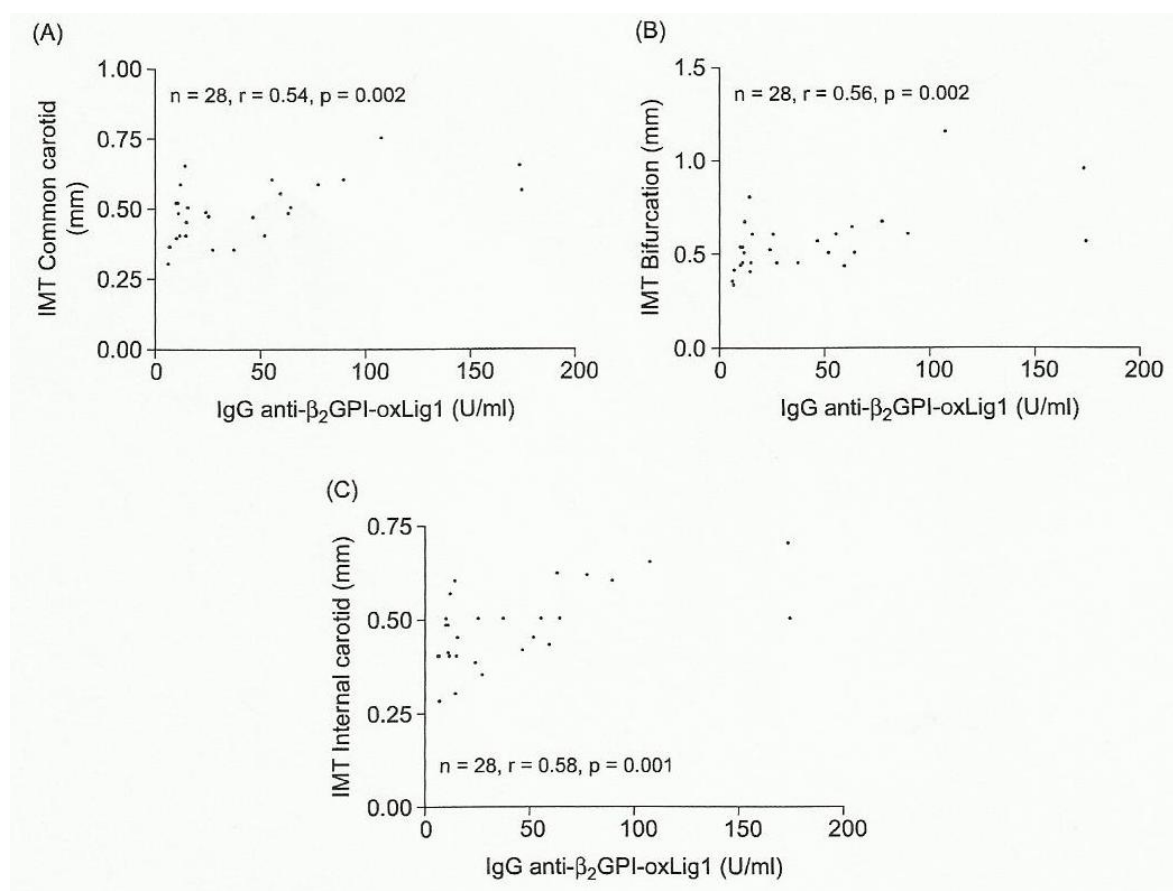


Figure 3. Relationship between IgG anti-β₂GPI-oxLig-1 and intima media thickness (IMT) of common carotid artery (A), carotid bifurcation (B) and internal carotid artery (C) in thrombotic APS patients.

3) DEFINITE EVIDENCE FOR ATHEROSCLEROSIS IN PAPS

Participants

The study was devised as a cross-sectional case double-control. To account for the occurrence of thrombosis, persistence of aPL and long-term warfarin intake of patients with PAPS, we enrolled a group of patients on long-term warfarin for vascular occlusions as a result of IT. Normal controls were recruited from hospital personnel undergoing annual health checks at the local Occupational Health Department and patients' friends. From January 2003, consecutive thrombotic PAPS patients and patients with IT attending the Coagulation Unit of the Antonio Cardarelli Hospital (Naples, Italy) were invited to participate in the study that was carried out in accordance with the revised Declaration of Helsinki, with approval of the

Ethics Board of the Hospital and written consent obtained from all participants. Exclusion criteria were acute or chronic hepatic, renal and lung disease and diabetes. As a result of obvious difficulties in recruiting thrombotic IT patients perfectly matched by age and gender with PAPS and normal controls, by January 2008 we still lacked nine matching IT patients. To overcome this impasse, our specialist nurse performed a search by age and gender of our database to locate those IT patients who had moved their follow up to different anticoagulant clinics within or without the town after our initial diagnosis: a letter detailing our study and an invitation to participate was sent to 16 matching IT patients from the database. The first nine who agreed and met exclusion criteria entered the study in compliance with our ethics approval. All participants underwent a questionnaire to review information on diabetes, liver, renal and lung disease, hypertension, menopause, oral contraception, hormone replacement therapy and a parental history of cardiovascular disease. Of our PAPS patients ($n = 60$), 11 were excluded: two developed SLE (one of gradual onset, one of abrupt presentation after IVF), one kidney cancer, two developed type 2 diabetes mellitus and six because they had miscarriages but not vascular occlusions. Of the IT patients ($n = 91$), 35 were excluded because they did not match PAPS patients, three were excluded because of chronic liver disease, two because of chronic obstructive pulmonary disease and two developed type 1 diabetes mellitus. The study, therefore, included 49 thrombotic PAPS patients (18 M, 31 F, mean age 37 ± 11 years) and an equal number of IT patients and controls subjects all perfectly matched by age and gender. All PAPS participants were on warfarin prophylaxis, three were on carbamazepine for post-ischemic epilepsy, two on beta-blockers, six on folic acid and one on angiotensin converting enzyme inhibitor. All IT patients were on warfarin prophylaxis, one on beta-blockers, seven on folic acid and two on angiotensin converting enzyme inhibitors. The control group included 34 healthy hospital personnel and 15 friends of patients. Neither patients nor controls were on lipid-lowering agents. A morning sample (after 14 h of fasting)

was taken for total-, HDL and LDL cholesterol and TG: healthy controls were also checked for genetic thrombophilia and aPL antibodies. Height and weight without shoes and garments were taken for BMI calculated as the ratio of weight in kilograms to height in square meters. Blood pressures were measured twice on one occasion, 1 min apart, with the participant seated and after 5 min of rest with the use of a standard mercury sphygmomanometer. Table 6 illustrates demographics of all participants.

Table 6. Demographics of participants in study groups.

	CTR	IT	PAPS
	(n=49)	(n=49)	(n=49)
Female (%)	31 (63)	31 (63)	31 (63)
Mean age (years)	37±11	37±11	37±11
Mean age 1 st thrombosis (years)	37±11	31±10	32±10
Heterozygote FVL	4	16	3
Heterozygote PT	2	12	5
AT deficiency	0	1	0
PC deficiency	0	6	0
f-PS deficiency	1	10	27
FVL + PT	0	4	0
<i>Thrombosis type</i>			
Arterial		7	12
Venous		41	34
Arterial + venous		1	3
<i>Thrombosis number</i>			
N = 1		39	29
N = 2		10	15
N = 3		0	4
N = 4		0	1
Current smokers (%)	12 (24)	13 (27)	18 (37)
<11/day	9	6	12
11-20/ day	3	5	6
>20/day	0	2	0
Ex-smokers	0	1	4
Menopausal	4	4	3
HRT	0	1	0
Oral contraception	5	0	0

CTR: control; IT: inherited thrombophilia; PAPS: thrombotic primary antiphospholipid syndrome; FVL: factor V Leiden; PT: prothrombin 20210; AT: antithrombin; PC: protein C; PS: protein S; HRT: hormone replacement treatment.

RESULTS

Clinical features and lipid profiles across groups

Table 7 summarizes the results of this section. PAPS and IT patients exhibited on average greater BMIs than controls (Table 2): a BMI greater than 30 was found in 10% (n=5) of PAPS, in 6% (n=3) of IT and 2% (n=1) of control participants. Systolic and diastolic blood pressures were similar across groups. Average total cholesterol was slightly lower in PAPS than other groups, although cholesterol higher than 200 mg/dL were present in 22% (n=11) of PAPS, in 28% (n=14) of IT and 6% (n=3) of CTR participants (p=0.01). Average HDL cholesterol was lower in PAPS and IT compared with controls whereas LDL cholesterol was comparable across the groups. In the same way, the average triglycerides concentration was higher in the PAPS and IT groups than in the control group.

Table 7. Clinical features of participants in study groups.

	CTR	IT	PAPS	p-value
	49	49	49	
Parental history of CVD	4	5	4	
BMI	23.4±2.4	25.4±3.5	25.3±3.2	0.001
Blood pressures				
Systolic (mm/Hg)	123±15	123±15	125±17	
Diastolic (mm/Hg)	77±7.9	78±7.1	80±9.6	
Pulse (mm/Hg)	45±9.8	45±9.8	45±11	
CHO (mg/dl)	181±29	181±36	177±34	
HDL (mg/dl)	56±14.2	40±15	37±10.8	<0.001
LDL (mg/dl)	109.6±28.5	99.23±27.4	96.5±31.27	0.08
TG (mg/dl)	83.1±29.2	108.8±44.2	106.2±54.8	0.01

IMT of carotid artery segments across groups

A comparison of the three groups under study revealed greater IMT of different carotid artery segments in PAPS than other groups (Table 8).

Table 8. Intima–media thickness of carotid artery segments across groups.

	CTR (n = 49)	IT (n = 49)	PAPS (n = 49)	p-value
Common carotid (mm)	0.416±0.085	0.448±0.096	0.487±0.123	0.004
Carotid bifurcation (mm)	0.481±0.108	0.521±0.093	0.556±0.162	0.013
Internal carotid (mm)	0.385±0.092	0.415±0.092	0.463±0.118	0.001

PAPS = primary antiphospholipid syndrome; IT = inherited thrombophilia; CTR: controls. Bonferroni's post hoc test; common carotid, PAPS vs CTR p=0.002; carotid bifurcation, PAPS vs CTR p=0.01; internal carotid, PAPS vs CTR p=0.001.

When we divided our participants into age tertiles, PAPS patients in the second and third tertiles showed greater IMT than in the other groups (Table 9). Plaques were uncommon in the PAPS group and occurred only in the third age tertile: 11% (2/17) of PAPS, 5.8% (1/17) of IT and 5.8% (1/17) of control participants.

Table 9 Intima-media thickness of carotid artery segments by age tertiles.

	Intima-media thickness of common carotid (mm)			
	CTR	IT	PAPS	P
1st tertile	0.347±0.030	0.386±0.064	0.406±0.090	0.054
2nd tertile	0.384±0.047	0.405±0.055	0.468±0.088	0.003
3rd tertile	0.496±0.074	0.528±0.085	0.581±0.119	0.030
	Intima-media thickness of carotid bifurcation (mm)			
1st tertile	0.412±0.078	0.479±0.083	0.438±0.081	0.154
2nd tertile	0.446±0.065	0.504±0.085	0.529±0.094	0.023
3rd tertile	0.557±0.113	0.564±0.093	0.693±0.171	0.004
	Intima-media thickness of internal carotid (mm)			
1st tertile	0.320±0.056	0.368±0.091	0.366±0.086	0.217
2nd tertile	0.374±0.083	0.375±0.061	0.460±0.076	0.003
3rd tertile	0.444±0.086	0.480±0.077	0.546±0.112	0.007

1st tertile: 16 patients (10 M, 6 F), mean age 26±4.4 years; **2nd tertile:** 16 patients (10 M, 6 F), mean age 36±2.7 years; **3rd tertile:** 17 patients (11 M, 6 F), mean age 49±8.5 years

Variables relating to IMT by univariate analysis were entered in a regression model to identify those independently related to IMT of different carotid segments: PAPS status and age emerged as the most powerful predictors for all carotid segments with triglycerides appearing as a strong predictor for the common carotid and female gender for the internal carotid (Table 10). A separate comparison of the two thrombotic groups showed greater CC ($p = 0.023$), B ($p = 0.04$) and IC ($p = 0.004$) in PAPS than IT after correction for the confounding effect of CHO, LDL and TG. However, the average number of thrombotic events was greater in PAPS than IT patients (1.52 ± 0.74 vs. 1.20 ± 0.41 , $p=0.01$) with a trend for greater IMT in patients with more thrombotic events (not shown). After inclusion of mean thrombotic events amongst the confounders, only IMT of the IC remained significantly greater in PAPS than IT patients ($p = 0.01$).

IMT predictors in PAPS

IMT of the common carotid correlated with age ($r=0.63$, $p<0.0001$), systolic ($r=0.63$, $p<0.0001$) and diastolic blood pressure ($r=0.50$, $p=0.0002$), IgG aCL ($r=0.37$, $p=0.009$) and IgG β_2 GPI titre ($r=0.45$, $p=0.001$) and number of occlusive events ($r=0.36$, $p=0.01$). Similarly IMT of the carotid bifurcation correlated with age ($r=0.75$, $p<0.0001$), systolic ($r=0.63$, $p<0.0001$) and diastolic blood pressure ($r=0.58$, $p<0.0001$), IgG β_2 GPI titre ($r=0.46$, $p=0.001$) and number of occlusive events ($r=0.31$, $p=0.02$). Finally IMT of the internal carotid correlated with age ($r=0.7$, $p<0.0001$), systolic ($r=0.54$, $p<0.0001$) and diastolic blood pressure ($r=0.54$, $p<0.0001$) and IgG β_2 GPI titre ($r=0.52$, $p=0.0003$). Both IgG aCL and IgG β_2 GPI correlated with age ($r=0.34$, $p=0.01$ and $r=0.5$, $p=0.0001$ respectively) whereas IgG β_2 GPI correlated with diastolic ($r=0.38$, $p=0.008$) and systolic ($r=0.38$, $p=0.009$) blood pressures. Given these interdependences, a multiple regression model identified age and diastolic blood pressure as independent predictors of CC IMT ($F=10.6$, $p=0.003$ and $F=4.6$, $p=0.04$ respectively), and age as an independent predictor of B ($F=26.5$, $p=0.0001$) and IC ($F=13.7$,

p=0.001) IMT. No IMT difference was noted in any carotid segment between nine PAPS patients with ischaemic stroke and nine PAPS patients with venous thrombosis matched by gender and age (2.7 ± 0.9 years) (not shown). PAPS patients with arterial occlusions showed higher mean diastolic pressure than PAPS patients with venous occlusions (84 ± 9 vs 77 ± 9 mmHg, p=0.01).

Table 10: Independent predictors of intima media thickness of carotid artery segments across groups by multivariate analysis.

Dependent variable: intima media thickness of common carotid			
Independent variables	β	SE	P
Parental history cardiovascular disease	0.017	0.021	0.401
Age	0.007	0.001	< 0.001
HDL cholesterol	0.000	0.000	0.997
Systolic blood pressure	0.000	0.001	0.933
PAPS	0.032	0.008	< 0.001
Triglycerides	0.000	0.000	0.015
Dependent variable: intima media thickness of carotid bifurcation			
Independent variables	β	SE	P
Age	0.008	0.001	< 0.001
HDL cholesterol	0.000	0.001	0.933
Systolic blood pressure	0.000	0.001	0.589
PAPS	0.039	0.011	0.001
Dependent variable: intima media thickness of internal carotid			
Independent variables	β	SE	P
Parental history cardiovascular disease	0.035	0.025	0.164
Age	0.006	0.001	< 0.001
HDL cholesterol	0.000	0.001	0.550
Systolic blood pressure	0.001	0.001	0.444
PAPS	0.038	0.010	< 0.001
Triglycerides	0.000	0.000	0.003
Sex	0.035	0.015	0.021

DISCUSSION

Patients with SLE have a 50-fold greater risk of developing cardiovascular disease in comparison to the general population (21) a phenomenon attributed to the development of premature atherosclerosis (22). A case-control study found increased IMT in SLE, partially explained by the presence of aPL (23) whereas aPL did not discriminate between SLE patients with and without carotid plaques (24). Moving this issue into PAPS, the first two studies in this chapter addressed whether aPL related to IMT in persistent carriers of aPL (thrombotic and non thrombotic) alongside the lipid profile and some haemostatic variables: this was appeared as a likely possibility given the enhanced the production of vWF, tissue factor and adhesion molecules from endothelial cells incubated in the presence of aPL (25-27).

Elevated plasma concentrations of vWF and FNG have been associated with thrombosis type and number in PAPS (28) and elevated concentration of adhesion molecules have been linked to the severity of APS (29). The univariate association between IgG aCL, vWF, FNG and PAI on the one side and IMT on the other strongly favour their involvement in the development of carotid atherosclerosis. Multivariate analysis showed that plasma FNG was an independent predictor of IMT. Elevated plasma FNG in combination with depressed fibrinolysis and enhanced coagulation activation contributes to accelerated fibrin turnover in PAPS (30). Depressed fibrinolysis and heightened fibrin turnover contribute to the persistence of fibrin deposition on endothelial cells, one of the initiating steps of atherosclerosis (31).

Most importantly, IgG aCL independently predicted the IMT of the different carotid segments examined. Outside the autoimmune setting aCL was an independent risk factor for angiographically documented atherosclerosis (32) and in young patients with peripheral vascular disease those (6) highlighting aCL as the possible major atherosclerotic risk factor.

Additionally, also plasma HC also ranked as an independent predictor of IMT in our patients. Elevated plasma HC is a risk factor for thrombosis (33) and elevated plasma HC

seems to be implicated in the thrombotic tendency of PAPS. In fact, patients with deep vein thrombosis who were found to have high aPL titres together with elevated plasma HC developed thrombosis at an earlier age than patients with elevated aPL alone or elevated HC alone (34). Similarly, in patients with PAPS, plasma HC had some impact on the number of occlusive events (35). Thus, plasma HC represents an additional risk factor contributing to the development of thrombosis and atherosclerosis in PAPS. Elevated HC and aPL share some pathogenic effects. HC may enhance tissue factor activity and PAI generation from the vascular endothelium in vitro (36, 37) whereas in vivo it is associated with elevated plasma levels of vWF and endothelin-1 (38-40), the same factors that are elevated in PAPS (28, 41, 42) particularly in patients with arterial disease (41, 42). Furthermore, elevated plasma HC and aPL enhance lipid peroxidation (43, 44) another process implicated in atherogenesis (45).

Indeed, oxidation of LDL plays an important role in atherosclerosis, as oxLDL may induce vasoconstriction, expression of adhesion molecules and cellular proliferation (46-48). On the other hand, immunisation of hypercholesterolemic rabbits with oxLDL suppresses atherosclerosis (49) therefore an immune response to oxLDL may modulate atherogenesis (50). An inverse relationship between oxLDL and IgG anti-oxLDL exists in the general population, and it was implied that anti-oxLDL helps to remove oxLDL from the bloodstream (51). However, this issue is controversial. One study described an inverse and independent relationship between anti-oxLDL titre and IMT of carotid arteries in healthy subjects (52), whereas, IgG-oxLDL titre positively associated with IMT of the CC artery in another study (53). That is, both the antigen and the corresponding antibody seem to have a relation with IMT. Similar conflicting information is available for SLE, where oxLDL appeared to be related to arterial thrombosis (54) or venous thrombosis (55) in SLE-related APS, whereas, more recently oxLDL related to disease activity (56).

The approach of our study is a step ahead of the oxLDL/anti-oxLDL system. A complex of β_2 GP with oxLDL and more specifically with the 7-ketocholesteryl-9-carboxynonanoate (oxLig-1) moiety from oxLDL and specific antibodies against this complex, IgG anti- β_2 GPI-oxLig-1, discriminated arterial disease in SLE related APS (11). The relation of this antigen/antibody system with IMT of carotid arteries in PAPS is unknown. Median plasma levels of the antigen, oxLDL- β_2 GPI complex, was higher in our control populations and lower in aPL subjects, whereas, the antibodies IgG anti-oxLDL- β_2 GPI and IgG anti- β_2 GPI-oxLig-1 were greatest in the PAPS group, though they did not identify patients with arterial thrombosis.

PONa showed several interesting relationships. In the control group, oxLDL/ β_2 GPI inversely related to PONa, indicating that as more LDL becomes oxidised, more oxLDL is buffered by β_2 GPI. This is likely due to an age related decrement of the antioxidant capacity of PON (57) given the lack of effect of oxLDL/ β_2 GPI on PONa in our in vitro experiments and the loss of correlation between oxLDL/ β_2 GPI and IMT of carotid segments after correction for age. We cannot discount the possibility that IgG anti- β_2 GPI-oxLig-1 appears as an attempt to clear oxLDL/ β_2 GP in a fashion similar to that described between oxLDL and IgG oxLDL in normal subjects (51). The data of our thrombotic control group partially replicate those of our normal control group and altogether they suggest that oxLDL/ β_2 GPI, IgG anti- β_2 GPI-oxLig-1 and anti oxLDL/ β_2 GPI might have a role in vascular ageing in normal people. The picture of the PAPS group mirrors that of the control groups. In fact, PONa negatively related to IgG β_2 GPI- oxLig-1, to IgG β_2 GPI and IgG aCL, in keeping with our hypothesis that PONa inhibition participates in APS related vascular damage (58). Here, we extend this concept, in that elevated IgG anti- β_2 GPI-oxLig-1 together with low PONa were positive and negative correlates of the IMT of carotid artery segments. Some aPL have inhibitory effects on PONa in SCID mice (59). With consideration to the small numbers of

our groups, we suggest the following sequence of events. In normal people, an age related decrease of PONa allows for oxidation of LDL that is buffered by β_2 GPI and eventually an antibody develops to clear this ternary complex. By contrast, in PAPS, aPL interference with PONa tilts the oxidant/antioxidant balance towards enhanced lipid peroxidation (44) that in turn contributes to arterial thickening. Although this study was not devised to demonstrating premature vascular damage in PAPS, it provides further evidence for PON and IgG anti- β_2 GPI-oxLig-1 implication in atherogenic pathways that may represent a target for therapeutic intervention.

In fact, atherosclerosis in PAPS may enhance the cardiovascular risk therefore further contributing to the morbidity of affected patients in most arterial districts (32, 60). Data from the Italian Antiphospholipid Registry calculated a 2.5% patient/year incidence of recurrent thrombosis often fatal (61); a prospective Spanish study demonstrated that 5.2% (7/133) of PAPS patients died of recurrent arterial occlusions (62); our own longitudinal study showed a 5.2% patient/year mortality rate for recurrent arterial thrombosis (63) and a Russian group recently reported a 17% 8-year vascular mortality for PAPS % (64). At variance from other case-control studies that lacked a control group with vascular occlusions and with a persistent risk factor such as genetic thrombophilia we demonstrate that the average IMT of our PAPS patients is significantly greater than that of IT and healthy controls. However, because of the wide age range, most of the difference of the IMT is found in the second and third age tertiles, as one would eventually expect. Indeed age was a significant predictor of IMT in all carotid segments considered. The only other study that compared PAPS patients with deep vein thrombosis to patients with thrombosis because of other causes (14) expressed atherosclerosis as plaques and the prevalence of which was 55% in PAPS and 75% in controls at the carotid level. Although not significantly different, this plaque prevalence is much greater than our 11% and than the 8% of the Spanish study, probably because of the high prevalence of other

risk factors for atherosclerosis described in that PAPS population (65). A high prevalence of atherosclerotic risk factors was also present in 28 PAPS patients from a Mexican study: their healthy controls were adequately matched for dyslipidaemia but not for hypertension and obesity: this might have had bearings on the IMT of 2.6 mm in the PAPS group vs. the 1.2 mm in the healthy group (66). Plaques were not defined in this study. Of the other two reports, the average IMT of 25 PAPS Spanish patients was not greater than healthy controls (65) whereas the average IMT of 14 Hungarian patients was greater than their controls (67). Interestingly, average total or LDL cholesterol in our PAPS patients were not elevated, rather average HDL cholesterol was low. In the mid 1990s, a case–control study found IgG aCL in 14.5% of patients younger than 50 years of age operated for atherosclerotic peripheral vascular disease. These, mostly female, had a lower prevalence of hyperlipidaemia/dyslipidaemia compared with those negative for IgG aCL (6). A possibility, not tested here, is that antibodies against HDL enhance HDL clearance contributing thus to the loss of the anti-atherogenic and anti-inflammatory properties of HDL (58). Indeed low grade inflammation characterizes thrombotic PAPS (68). The lack of hyperlipidaemia strengthens the role of aPL as a unique cardiovascular risk factor in thrombotic PAPS: herein IgG aCL and IgG β_2 GPI correlated with IMT segments although by multivariate analysis only the age effect became prevalent. A notable observation derived from the comparison of the two thrombotic groups is that the average number of thromboses in the PAPS group accounted for the greater IMT of the carotid segments of PAPS compared with IT; in fact after correction for this confounding only IMT of the IC remained significantly greater in PAPS than in IT. In other words the number of occlusive events may affect IMT.

In conclusion, we provide the first evidence that premature atherosclerosis as defined by IMT occurs in thrombotic PAPS over 30 years of age; however, our design and numbers cannot provide an answer as to whether premature atherosclerosis contributes to an increased

risk of thrombosis, and the correlation between IMT and number of vascular occlusions may even suggest the opposite. Whichever the case, warfarin may prevent thrombosis, but has no effect on atherosclerosis, the progression of which should be prospectively assessed and eventually managed by specific therapeutic agents to prevent additional vascular morbidity.

CHAPTER IX

CONCLUSION

Coagulation, oxidation, inflammation, immune activation and nitration in primary antiphospholipid syndrome: the atherogenic link

The possibility that some risk factors for thrombosis and atherosclerosis in the general population (1-3) would behave as risk factors thrombosis and atherosclerosis in PAPS proved true. In Chapter I plasma vWF and FNG were differentially associated with arterial and venous occlusions regardless of sex whereas in Chapter VIII plasma FNG was an independent predictor of IMT of carotid arteries alongside IgG aCL. At variance the data on fibrinolytic variables were gender related, in that female PAPS showed blunted tPA release and higher mean plasma PAI compared to male patients. Epidemiological surveys in the general population have demonstrated that females show an age related increase of plasma PAI compared to males (4) whereas the opposite occurs for tPA (5), suggesting that aPL may emphasize the age and sex dependency of these fibrinolytic variables in PAPS. Moreover elevated mean plasma tPA and PAI in patients with a history of thrombosis is a risk factor for recurrences (6) and elevated PAI is a risk factor for atherosclerosis (3): indeed Chapter VIII shows that plasma PAI associated with IMT of carotid arteries though only by univariate analysis. Overall the results on haemostatic and fibrinolytic variables indicate that PAPS patients and persistent carriers of aPL in the absence of thrombosis are in a state of accelerated fibrin turnover and fibrin deposition on the vessel wall is a recognized early phenomenon in atherogenesis (7).

Thrombotic PAPS patients and persistent carriers of aPL revealed an unusual lipid profile characterised by low plasma concentration of HDL and of LDL (8). In PAPS, the presence of antibodies against HDL may induce the formation of an antigen/antibody complex that is

removed by phagocytic cells allowing for reduced HDL; moreover in vitro studies have shown that aPL are able to induce a dose dependent inhibition of the activity of PON (9), one of the enzymes that prevents the oxidation of LDL. Whichever the mechanism, in isolation or in combination, there will be increased oxidation of LDL, independent of the plasma concentration of LDL, with release of F2-isoprostane, a powerful vasoactive agent increased in PAPS (10) and a sensitive marker of lipid peroxidation, an early step in atherogenesis (11).

In an antioxidant attempt to quench LDL oxidation, β_2 GPI binds to LDL in a stable oxLDL/ β_2 GPI complex (12): the plasma concentration of oxLDL/ β_2 GPI was elevated in PAPS where it was independently predicted by IgG anti-HDL (8). Interestingly the inflammatory marker CRP negatively correlated to HDL and ApoAI suggesting that loss of antioxidant capacity allows the emergence of low grade inflammation pursuant the concept that oxidation and inflammation are parallel processes in PAPS: indeed not only the plasma concentration of oxLDL- β_2 GPI but also that of CRP was elevated in PAPS (8).

The low grade inflammation in PAPS was confirmed in another study on the same cohort of patients where the elevated plasma concentrations of oxLDL- β_2 GPI and CRP were matched by that of SAA, another inflammatory marker and by sCD14 and NPT, immune activation markers. Of these markers, SAA related to the number of thrombosis, but NPT and sCD14 were associated to the number of arterial thrombosis (13). Soluble CD14 and NPT are respectively shed and released by monocytes activated by interferon gamma of T helper cell origin (14). Monocytes are central to the vascular pathogenesis of PAPS: their exposure to aPL in the circulation may promote tissue factor expression that enhances systemic coagulation possibly explaining arterial thrombosis (15); moreover, in the circulation monocytes encounter oxLDL, oxLDL- β_2 GPI complexes, IgG-oxLDL- β_2 GPI immune complexes and eventually phagocytose them either via scavenger receptors or via Fc gamma

receptors, during which process they migrate within the intima of the arterial wall, again an early atherogenic step as the loss of the biological activity of NO (16).

The plasma concentration of NO_2^- , the “vasodilator” metabolite of NO was markedly reduced in persistent non thrombotic carriers of aPL and in PAPS, but particularly in patients with multiple occlusions, whereas the plasma concentration of NO_3^- , the “inflammatory” metabolite of NO was associated with arterial disease (17). Linking with previous data, these PAPS patients also showed a moderate degree of nitrative stress, measured as plasma NT, and plasma NT was independently predicted by CRP, the same marker associated with oxidation (8). Therefore we have a scenario where increased fibrin turnover, even in orally anticoagulated PAPS patients (18), oxidative and nitrative stress, loss of the biological activity of NO, low grade inflammation and immune activation are all interconnected. Given that these phenomena occur at the early stages of atherosclerosis in the normal population, it will be of no surprise that they contribute to premature atherosclerosis in PAPS as demonstrated (19).

Future directions

The current therapeutic strategy in thrombotic PAPS is to maintain indefinite oral anticoagulation to prevent thrombotic recurrences that unfortunately do happen, particularly in male patients with strong lupus anticoagulants and high positive aPL (20). At present we do not know how to manage PAPS related atherosclerosis. Control of modifiable risk factors is intuitive but the progression of heart valve lesions in PAPS is worrying (21) and warrants clinical intervention. Treatment may be directed at managing the effects of aPL antibodies or at decreasing aPL titres. A pilot study with the use of probucol, a drug with antioxidant properties showed significant improvements in fibrinolysis and decreased urinary thromboxane, isoprostane and NO metabolites after three weeks treatment (22). Given their positive effects on the immune system and on the vascular endothelium statins may be suitable candidates within the context of a clinical trial (23). Probucol was non inferior to

statins in a head to head trial aimed at regressing atherosclerosis in a general population over 60 (24). Attempts at reducing plasma levels of aPL via B-cell depletion in PAPS were unsuccessful (25) and perhaps plasma cell depletion coupled with antigen manipulation/vaccination may represent a breakthrough for antibody mediated disorders (26).

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REFERENCES TO CHAPTER I

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